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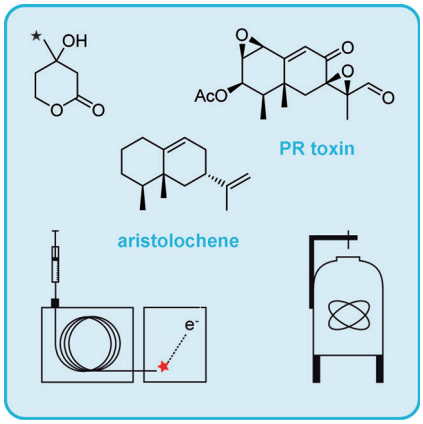
Complex bouquet: A recently developed method for trace analyses combining the advantages of GC-MS and ^{13}C NMR spectroscopy was applied to investigate the volatiles of *Penicillium roqueforti*. Besides the main compound, aristolochene, several side products of aristolochene synthase and downstream oxidation products en route to PR toxin were identified, giving insight into the biosynthetic pathway.



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PR Toxin Biosynthesis in *Penicillium roqueforti*

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PR Toxin Biosynthesis in *Penicillium roqueforti*

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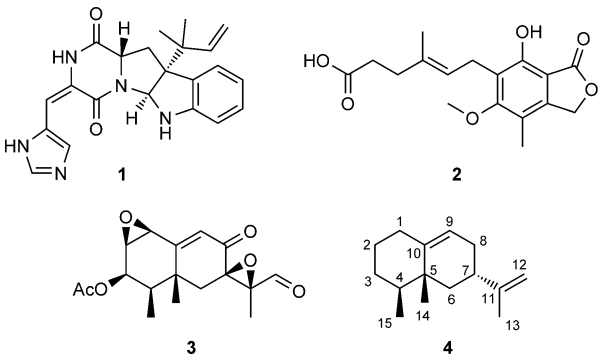
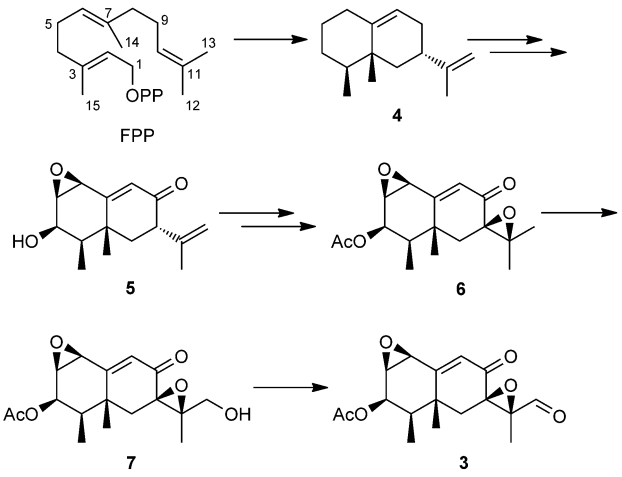
The ascomycete *Penicillium roqueforti* is well-known for its industrial application in the production of blue cheeses. Although the fungus is used in the food industry, a variety of bioactive secondary metabolites, some of them toxic to humans, from this organism are also known; they include the neurotoxin roquefortine C (**1**, Scheme 1)[1] and the immunosuppressant mycophenolic acid (**2**).[2] Moreover, mycotoxins of sesquiterpenoid origin have been characterized, out of which the oxygenated PR toxin (**3**)[3,4] exhibits the highest bioactivity.



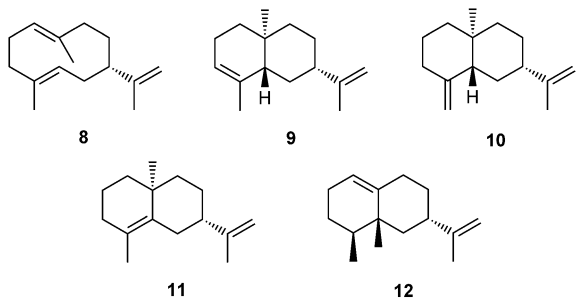
Scheme 1. Structures of secondary metabolites from *P. roqueforti*: roquefortine C (**1**), mycophenolic acid (**2**), PR toxin (**3**), and aristolochene (**4**).

The biosynthesis of the PR toxin starts with the formation of aristolochene (**4**, Scheme 2) from the universal sesquiterpene precursor farnesyl diphosphate (FPP) in a reaction catalyzed by aristolochene synthase.[5,6] The suggested sequence for the following oxidation steps towards **3** was mainly based on the purification and identification of biosynthetic intermediates from culture extracts. The first known of these intermediates is eremofortin B (**5**),[7] which arises from **4** by an unknown series of oxidation steps. All earlier intermediates might have been overlooked because of their relatively high volatility, which can easily result in loss of material during culture extract concentration steps. The intermediate **5** might subsequently be acetylated and epoxidized to form eremofortin A (**6**). Further hydroxylation gives eremofortin C (**7**), which is converted into PR toxin by eremofortin C oxidase.[7] No clustered genes for this pathway have been identified, and other than aristolochene synthase and eremofortin C oxidase, no other enzymes involved in the biosynthesis of PR toxin have been characterized until now.



Scheme 2. Proposed biosynthetic pathway from aristolochene (**4**) to PR toxin (**3**).

The initial formation of aristolochene from FPP by aristolochene synthase proceeds via cationic intermediates and the neutral intermediate germacrene A (**8**, Scheme 3).[5,6,8] The cyclization mechanism has been analyzed in detail in incubation experiments with aristolochene synthase and isotopically labeled FPP; this also gave insights into the stereochemical course of the reaction.[9–11] An interesting aspect is the highly stereocontrolled transformation of the terminal *Z* methyl group of FPP (C-13, cf. Scheme 2) to afford the *exo*-methylene group of **4** (C-12, cf. Scheme 1).[10] Moreover, the aristolochene synthases from *P. roqueforti* and from *Aspergillus terreus* have been crystallized.[12–14] Known aristolochene synthase side products that are specifically found in *P. roqueforti* strains capa-



Scheme 3. The neutral intermediate germacrene A (**8**) and known aristolochene synthase side products.

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ble of producing the PR toxin are germacrene A (**8**), α-selinene (**9**), and valencene (**12**).[15] Altered product distributions have been observed from mutant enzymes with specific switches of active-site residues.[16–20]

The closed-loop stripping apparatus (CLSA) is a powerful tool for investigating the volatile bouquets of microorganisms because even trace components can be identified. Furthermore, the method does not risk the loss of volatile material because no concentration steps are required. Analyzing CLSA headspace extracts by GC-MS often leads to the identification of numerous compounds from complex mixtures of natural products through comparison of mass spectra and retention indices with literature data. Major drawbacks of the method include difficulties in the identification of compounds that co-elute from the GC column, especially if a minor component is masked by large quantities of another one. Secondly, correct structure assignment can be difficult if several stereoisomers of a compound are possible because diastereomers often elute at similar retention times and their mass spectra usually share great overall similarity. For unambiguous identification, structure elucidation through NMR techniques would provide the necessary information, but the amounts of the analytes in the CLSA headspace extracts are usually too low and the compound mixtures too complex for direct NMR analysis.

Recently, however, we described a new analytical method that combines the advantages of the CLSA/GC-MS technique (detection sensitivity and efficiency of gas-chromatographic separation) with those of NMR spectroscopy (detailed structural information). For this purpose, feeding of a ^{13}C-labeled terpene precursor was performed to enhance specific ^{13}C NMR signals of the terpenes in CLSA extracts strongly even at low incorporation rates, thereby allowing for direct analysis through ^{13}C NMR spectroscopy.[21] Together with the valuable information obtained by GC-MS (mass spectrum and retention index), the additional information provided by ^{13}C NMR can be of use for the assignment of the relative stereochemistry to a molecule, when NMR data for the different stereoisomers are available from the literature. Furthermore, such experiments can be used to elucidate the stereochemical course of a particular terpene cyclization. Here we have adopted this methodology to analyze side products and intermediates of the aristolochene and PR toxin biosynthetic pathways for the identification of previously unidentified compounds and have demonstrated that the stereochemical course of the initial cyclization of FPP to the neutral intermediate **8** during cyclization to **4** can also be followed.

The volatiles emitted by *P. roqueforti* NRRL 849 agar plate cultures were collected by use of the CLSA technique. An agar plate was kept in a closed vessel for 24 h, during which an air stream was continuously directed over it and then through a charcoal filter containing 5 mg of charcoal. The filter was extracted with dichloromethane (30 μL), and the extracts were analyzed by GC-MS. The region of the total ion chromatogram containing the sesquiterpenes of a representative extract from *P. roqueforti* is depicted in Figure 1. Full results of the analyses are reported in Table S1 in the Supporting Information, and a full-length chromatogram is depicted in Scheme S1.

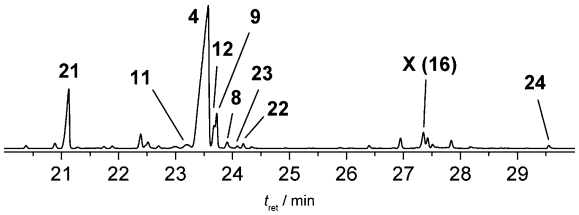
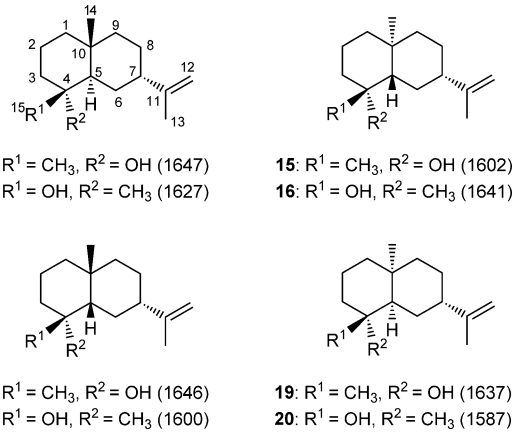


Figure 1. Total ion chromatogram of a representative headspace extract from *P. roqueforti* NRRL 849 grown on M90 agar plates.

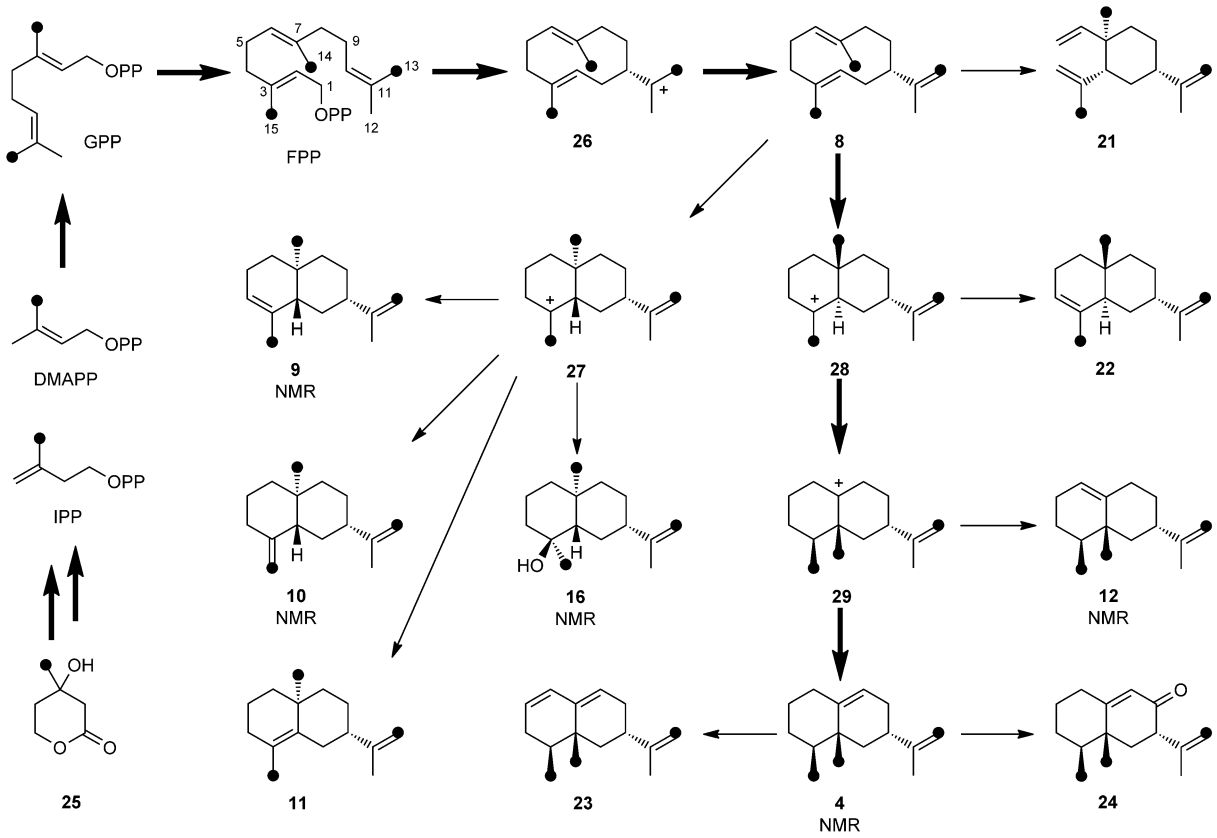
Several sesquiterpenes were identified through their mass spectra and retention indices, with aristolochene (**4**) the most prominent. Moreover, the known side products **9**, **11**, and **12** were found. Germacrene A (**8**) was also present, together with β-elemene (**21**, cf. Scheme 5, below), which arises through thermal Cope rearrangement of **8** in the GC injection port.[22] In addition, the sesquiterpenoids 7-*epi*-α-selinene (**22**) and nootkatene (**23**) could unambiguously be identified by comparison of mass spectra and GC retention indices with literature values; neither compound has ever been described from the aristolochene synthase. A eudesmane-type alcohol **X** was also tentatively identified from its mass spectrum, but because of contradictory retention index literature data none of the eight possible stereoisomers could be assigned to **X**. In one systematic study the retention indices of all eight stereoisomers on a DB-1 column were reported (summarized in Scheme 4);[23] they suggest that one of the isomers **13**, **16**, **17**,



Scheme 4. The eight possible diastereomers of eudesmane-type alcohol **X**. Retention index literature data on a DB-1 column are given in brackets.[23]

or **19** might represent **X** ($I = 1654$ on a HP-5MS column), but these data were not sufficient for unambiguous structural assignment. Furthermore, an oxidized sesquiterpene ($I = 1757$) with a molecular ion at *m/z* 218 and a mass spectrum almost identical to that of neopetasone ($I = 1733$[24]) was tentatively identified as an epimer of this compound.

Feeding experiments with a ^{13}C-labeled terpene precursor were conducted to establish the nature of **X**. For this purpose, [*methyl*-^{13}C]mevalonolactone (**25**, Scheme 5) was synthesized according to a procedure that was recently developed in our



Scheme 5. Unified biosynthetic scheme for aristolochene (**4**) and the observed side products. The main pathway to aristolochene is highlighted with bold arrows. The ^{13}C-label is indicated as black dots. Compounds that were identified by ^{13}C NMR spectroscopy are indicated.

laboratory[25] and fed to agar plate cultures of *P. roqueforti*. This labeled precursor is transformed through the mevalonate pathway into [13,14,15-$^{13}C_3$]FPP, which is further converted by the aristolochene synthase into sesquiterpene hydrocarbons (Scheme 5). Analysis of the CLSA headspace extracts by ^{13}C NMR and DEPT spectroscopy established its efficient incorporation into the sesquiterpenes (Figure 2). Three major peaks at $\delta = 15.7$, 18.1, and 108.3 ppm could immediately be assigned to two methyl groups and one olefinic methylene group of **4** (Figure 2A and Table 1). This finding fully corroborated the previously reported stereochemical course of the terpene cyclization with the terminal *Z* methyl group of FPP (C-13) being converted into the olefinic methylene carbon of **4** (C-12).[10] The other 12 carbons of **4** with the natural ^{13}C abundance were also observable (marked by asterisks).

Further signals represented other less abundant sesquiterpenes identified by GC-MS in the headspace extracts. Sets, each of two signals for methyl groups and one for an olefinic methylene group, for compounds **9** and **12** were found (Figure 2B and C, Table 1). Furthermore, β-selinene (**10**) was detected by ^{13}C NMR spectroscopy (set of two signals for olefinic methylene groups and one signal for a methyl group). This compound was masked in the GC-MS analysis by the coeluting main component aristolochene, thus giving a striking example of the potential of the combined analytical methods.

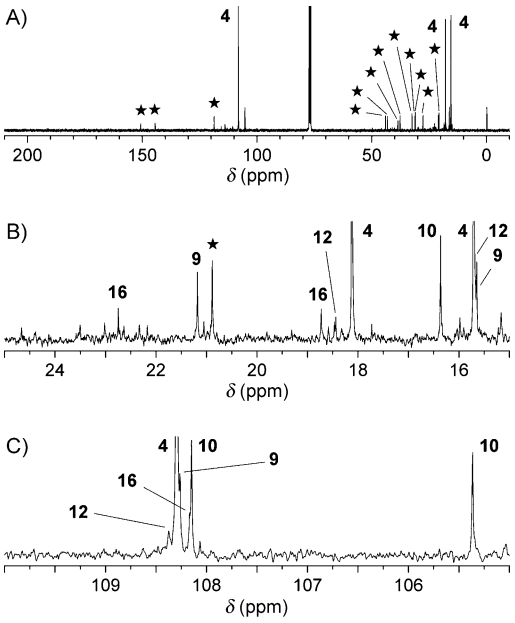


Figure 2. ^{13}C NMR spectrum of a representative headspace extract from *P. roqueforti* NRRL 849 grown on M90 agar plates supplemented with 2 mM [*methyl*-^{13}C]mevalonolactone (**25**).

Table 1. Chemical shifts observed after feeding of [*methyl*-^{13}C]mevalonolactone to agar plate cultures of *P. roqueforti* NRRL 849.

Compound	%	$I^{[a]}$	I (lit.)$^{[b]}$	C-12$^{[c]}$	C-13$^{[c]}$	C-14$^{[c]}$	C-15$^{[c]}$	Ref.
β-elemene (**21**)	12.7	1392	1389[24]	–	–	–	–	–
selina-4,11-diene (**11**)	1.6	1476	1475[24]	–	–	–	–	–
aristolochene (**4**)	71.2	1487	1487[26]	108.3 (108.3, CH_2)	n.d. (20.9, CH_3)	15.7 (15.7, CH_3)	18.1 (18.1, CH_3)	[24]
β-selinene (**10**)	n.d.	n.d.	1489[26]	108.1 (108.1, CH_2)	n.d. (21.0, CH_3)	16.3 (16.3, CH_3)	105.4 (105.3, CH_2)	[27]
valencene (**12**)	3.4	1493	1496[26]	108.4 (108.3, CH_2)	n.d. (20.8, CH_3)	15.7 (15.7, CH_3)	18.4 (18.4, CH_3)	[28]
α-selinene (**9**)	5.0	1495	1498[26]	108.2 (108.2, CH_2)	n.d. (20.9, CH_3)	15.6 (15.6, CH_3)	21.2 (21.2, CH_3)	[27]
germacrene A (**8**)	1.4	1504	1508[26]	–	–	–	–	–
nootkatene (**23**)	0.4	1514	1517[26]	–	–	–	–	–
7-*epi*-α-selinene (**22**)	0.7	1517	1520[26]	–	–	–	–	–
eudesma-11-en-4α-ol (**16**)	3.0	1654	1656[29]	108.2 (108.1, CH_2)	n.d. (21.0, CH_3)	18.7 (18.6, CH_3)	22.7 (22.6, CH_3)	[30]
7-*epi*-neopetasone (**24**)	0.5	1757	n.a.	–	–	–	–	–

[a] Retention index determined from a homologous series of alkanes; n.d. = not detected (compound masked by large quantities of **4**). [b] Literature data obtained with the same or a similar GC column; n.a. = not available. [c] Observed chemical shifts in the ^{13}C NMR spectroscopic analysis of the CLSA extract obtained after feeding of [*methyl*-^{13}C]mevalonolactone are given in parts per million and referenced to $CDCl_3$ ($\delta = 77.01$ ppm). Literature values and structural information obtained by DEPT spectroscopy are given in parentheses; n.d. = not detected (these ^{13}C NMR signals would have been expected for a different stereochemical course of the cyclization of FPP to germacrene A; cf. further explanation in the text).

Moreover, the stereochemistry of the eudesmane-type sesquiterpene alcohol **X** was revealed from an additional set of peaks in the ^{13}C NMR spectrum, at chemical shifts of $\delta = 18.7$, 22.7, and 108.2 ppm. Comparison with literature NMR data established the *trans* fusion of the six-membered rings. The chemical shift of C-14 (cf. Scheme 4 for carbon numbering of the eudesmane skeleton) ranges from $\delta = 18.3$ to $\delta = 18.7$ ppm for all of the *trans*-fused structures intermedeol (**13**), paradisiol (**14**), neointermedeol (**15**), and eudesma-11-en-4α-ol (**16**), but is found in the range of $\delta = 28.9$ to $\delta = 30.7$ ppm for all of the *cis*-fused eudesmane sesquiterpenoids 7-*epi*-amiteol (**17**), 5-*epi*-paradisiol (**18**), 5-*epi*-neointermedeol (**19**), and amiteol (**20**, Table 2).[23] For the *trans*-eudesmane alcohols, the chemical shift of C-15 is around 22.4 ppm for **13** and **16** and around 30.0 ppm for **14** and **15**; this, together with the coinciding signals of C-12, established the structure of **X** as that of eudesma-11-en-4α-ol (**16**).

Table 2. ^{13}C NMR data for the unknown alcohol **X** and comparison with those for the eight stereoisomers of eudesmane-type alcohols.[23]

		trans-Decalin derivatives				*cis*-Decalin derivatives			
Carbon	**X**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
C-14	**18.7**	18.4	18.3	18.7	**18.6**	30.5	30.7	28.9	29.5
C-12	**108.2**	110.7	110.4	108.3	**108.1**	108.0	107.9	109.1	107.6
C-15	**22.7**	22.2	29.8	30.2	**22.6**	31.2	31.2	30.3	31.2

The biosynthetic formation of all identified sesquiterpenes can be explained by several intermediate cations along the pathway to aristolochene (Scheme 5). Diphosphate abstraction from FPP and initial 1,11-cyclization give the (*E*,*E*)-germacradienyl cation, which is stereospecifically deprotonated at C-13 (FPP numbering) to yield germacrene A (**8**). Reprotonation and concomitant cyclization gives access to the *trans*-configured eudesmane-type cations **27** and **28**. α-Selinene (**9**), β-selinene (**10**), selina-4,11-diene (**11**), eudesma-11-en-4α-ol (**16**), and 7-*epi*-α-selinene (**22**) arise directly from **27** or **28**, variously by loss of a proton or by capture with water. Cation **28** can undergo a concerted 1,2-hydride shift with a methyl migration to afford **29**, thus giving access to aristolochene (**4**) and valencene (**12**) by alternative deprotonation modes. Nootkatene (**23**) and 7-*epi*-neopetasone (**24**) are oxidation products of aristolochene.

The incorporation of the label from [*methyl*-^{13}C]mevalonolactone into **4** and the side products that could be detected by ^{13}C NMR spectroscopy [into the sp^2 methylene carbon of the isopropenyl group and two additional carbons (usually two methyl groups; only in the case of β-selinene was a second olefinic methylene group labeled)] was observed in all cases. This observation is in full agreement with the formation of all sesquiterpenes via germacrene A (**8**).

The biosynthetic scheme also gives a good reason for the nature of the neopetasone epimer. Its abundance in the headspace extracts was unfortunately too low for detection by ^{13}C NMR spectroscopy in the labeling experiment, thus showing the limitations of the method. However, the only logic structure for this compound is that of 7-*epi*-neopetasone (**24**) because it can arise directly from the main compound **4** by oxidation. Its presence, together with the identification of nootkatene (**23**), gives an interesting insight into the subsequent steps from **4** in the PR toxin biosynthesis. Oxidation of aristolochene can happen in divergent biosynthetic pathways that eventually lead to the same oxidized intermediate eremofortin B (**5**).

In summary, our newly developed trace analytical method based on capturing volatiles on charcoal by use of a CLSA with concomitant feeding of a ^{13}C-labeled precursor, with monitoring by GC-MS and ^{13}C NMR analysis, combines all advantages of each of the used techniques. Firstly, trapping of volatiles by CLSA does not risk loss of volatile material. Secondly, GC-MS

analysis of the obtained headspace extracts is highly sensitive and has a high separation power. Its major drawback, its inability to provide precise structural information about terpenes with unknown mass spectra and/or retention indices, was resolved by application of ^{13}C NMR spectroscopy. This technique is not usually suitable for excessively complex headspace extracts containing many different compounds, with some of these present in amounts too low for detection, but in a feeding experiment with a ^{13}C-labeled precursor such as **25** the ^{13}C NMR spectrum can be simplified because only terpenes will be observable and only three indicative carbons for each sesquiterpene will be labeled. Furthermore, the signals for the labeled carbons are strongly enhanced even in cases of low incorporation rates. The new method was successfully applied in the detection of previously unrecognized side products and intermediates of the PR toxin biosynthetic pathway, and in addition gave insight into the stereochemical course of terpene cyclization through the action of aristolochene synthase. Future studies in our laboratory will include the further development and application of the analytical method for investigations of terpene biosynthetic pathways.

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Keywords: aristolochene · biosynthesis · isotopic labeling · PR toxin · terpenes · volatiles

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