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Harzianone Biosynthesis by the Biocontrol Fungus *Trichoderma*

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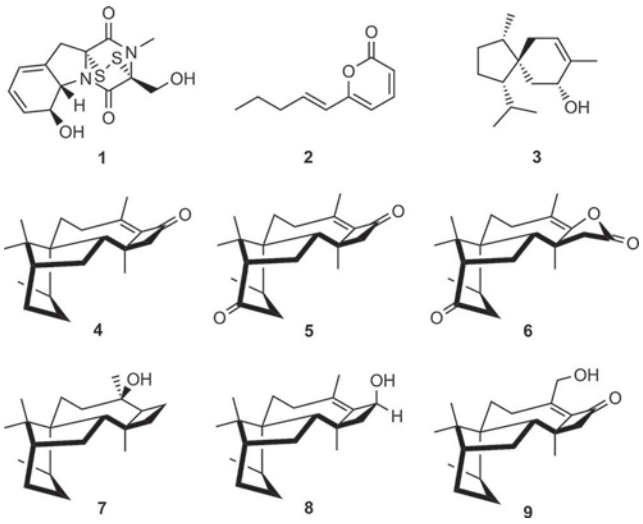
Analysis of the volatile terpenes produced by seven fungal strains of the genus *Trichoderma* by use of a closed-loop stripping apparatus (CLSA) revealed a common production of harzianone, a bioactive, structurally unique diterpenoid consisting of a fused tetracyclic 4,7,5,6-membered ring system. The terpene cyclization mechanism was studied by feeding experiments using selectively ^{13}C- and ^{2}H-labeled synthetic mevalonolactone isotopologues, followed by analysis of the incorporation patterns by ^{13}C NMR spectroscopy and GC/MS. The structure of harzianone was further supported from a $^{13}C,^{13}C$ COSY experiment of the in-vivo-generated fully ^{13}C-labeled diterpene.

Introduction

Fungi of the genus *Trichoderma* are widespread in soil and wood habitats and are known for their opportunistic avirulent plant beneficial attributes.[1] They are used as environmentally friendly biocontrol agents because of their positive effect on root growth and development, crop productivity, and nutrient uptake.[2] One aspect in the complex mechanisms of the *Trichoderma* plant–pathogen interaction is the ability of *Trichoderma* to parasitize other fungi, thereby protecting the plant from harmful phytopathogens.[3] This ability can be attributed to the production of cellulose- and chitin-degrading enzymes and the production of diverse bioactive secondary metabolites.[4] A well-known example is the diketopiperazine gliotoxine (**1**, Scheme 1) that was first isolated from *Gliocladium fimbriatum*, a fungus that was later reclassified as *Trichoderma virens*.[5] Gliotoxine is an immunosuppressive, toxic, antimicrobial compound that plays an important role in pathogen defense.[6] Fungi are also known as producers of volatiles with often distinct functions.[7] A series of volatile 2*H*-pyran-2-ones, such as **2**, were isolated from *Trichoderma*,[8] which exhibit growth inhibitory properties against phytopathogenic fungi including *Aspergillus*, *Botrytis*, *Rhizoctonia*, *Sclerotinia*, and *Pyrenochaeta*.[8c,d,9] A volatile sesquiterpene alcohol that is frequently released by *Trichoderma* is tricho-acorenol (**3**), which was first identified from culture extracts of *Trichoderma koningii*.[10] Its absolute configuration was deduced by enantioselective synthesis,[11] and its biosynthesis was addressed by feeding experiments with isotopically labeled precursors.[12] Furthermore, a series of structurally unique and biosynthetically related diterpenes represented by harzianone (**4**),[13] harziandione (**5**)[14] and trichodermaerin (**6**)[15] were isolated from *Trichoderma*. Notably, **5** and the related diterpenoids **7**–**9** were recently isolated from a *Trichoderma* symbiont of the taxane-producing plant *Taxus baccata*.[16] Diketone **5** is a strong antifungal compound that is active against the plant pathogenic fungus *Sclerotium rolfsii*,[17] whereas ketone **4** lacks such antifungal activity but exhibits activity against *Escherichia coli* and *Staphylococcus aureus* and in a brine shrimp (*Artemia salina*) toxicity assay.[13] The absolute configuration of **4** was determined by comparison of experimental and calculated electronic circular dichroism (ECD) spectroscopic data,[13] but so far no studies regarding the biosynthesis of this unique tetracyclic diterpene have been conducted. We recently developed a method that combines feeding of ^{13}C-labeled mevalonolactones and capturing of the resulting labeled volatile terpenes by collection with a closed-loop stripping apparatus (CLSA), which allows for direct analysis of the headspace extracts by ^{13}C NMR (CLSA-NMR).[18] This method is especially powerful if the biosynthesis of volatile terpenoids is



Scheme 1. Structures of known secondary metabolites from *Trichoderma*.

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to be addressed, as the classical method of liquid culture extraction and compound isolation in preparative amounts can be significantly hampered by compound loss during solvent evaporation steps. Furthermore, biosynthetic investigations with our CLSA method can be performed with only a single agar plate culture and thus require only small amounts of the expensive ^{13}C-labeled precursors. Here, we present our insights into harzianone (**4**) biosynthesis by application of this method to the fungus *Trichoderma*.

Results and Discussion

The volatile terpenes released by seven fungi of the genus *Trichoderma* were analyzed by use of a CLSA (Figures 1 A and S1).[19] In all headspace extracts, tricho-acorenol (**3**) was found as the main constituent, except for *Trichoderma atroviride*, which produces large amounts of **2**. Furthermore, all seven strains produced a compound with a mass spectrum that suggested the structure of an oxidized diterpene hydrocarbon, as indicated by a molecular ion at *m/z* 286 (Figure 1 B), but the mass spectrum was not included in our mass spectral libraries, preventing instantaneous compound identification. As production of this compound was the highest in *Trichoderma* sp. 34, this strain was chosen for all further experiments.

In order to identify the detected compound, its isolation by solvent extraction of agar plate cultures was attempted several times, but the production was too low to obtain sufficient material for structure elucidation by NMR. Therefore, feeding experiments with (2,3,4,5,6-$^{13}C_5$)-mevalonolactone[18f] ((2,3,4,5,6-$^{13}C_5$)-**10**) were carried out in which all five carbons ending in the terpene monomers dimethylallyl diphosphate (DMAPP, **11**) and isopentenyl diphosphate (IPP, **12**) were ^{13}C labeled. In a typical experiment, 10 mg of the labeled compound were fed to a standard agar plate culture (ca. 30 mL of medium), followed by collection of volatiles by CLSA for the next seven days. The charcoal filter was extracted with 50 µL C_6D_6 every day, and the collected extracts were directly used for further analysis. GC/MS analysis revealed an increased production of the diterpenoid due to the administration of the terpene precursor and a high incorporation of labeling (91 % incorporation rate, Figure S2). The content of the labeled diterpene in the sample was sufficient for recording a ^{13}C,^{13}C COSY spectrum that suggested the structure of harzianone (**4**, Figure 2). As a few crosspeaks for correlations to quaternary carbons were missing, the isolation of unlabeled **4** was re-attempted from a large number of agar plates (100 plates), resulting in the isolation of 3.8 mg of pure **4**. Its structure was confirmed by ^{1}H, ^{13}C, ^{13}C DEPT-135, ^{1}H,^{1}H COSY, ^{1}H,^{13}C HSQC, ^{1}H,^{13}C HMBC, and ^{1}H,^{1}H NOESY and comparisons to recorded literature data (Figure S4–S10).[13,16]

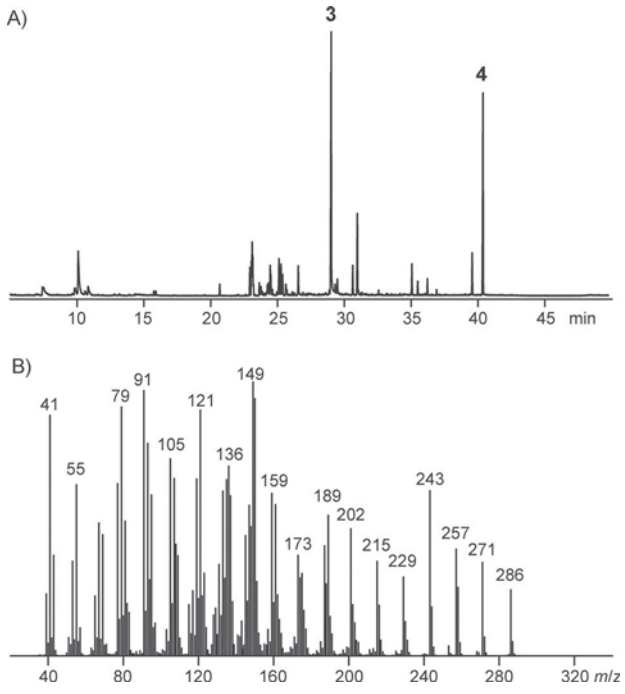


Figure 1. A) Representative total ion chromatogram of the headspace extract of *Trichoderma* sp. 34 and B) EI mass spectrum of harzianone (**4**).

The terpene cyclization mechanism of **4** was studied by feeding of a series of synthetic ^{13}C-labeled mevalonolactones (**10**).[18e] Three different possibilities for the fold of the diterpene precursor geranylgeranyl diphosphate (GGPP, **13**) could explain the formation of the harzianone backbone (Scheme 2 A). One of these folds, as shown in I-**4**, resembles the most straightforward arrangement, whereas the GGPP cyclization mechanisms for the substrate folds in II-**4** and III-**4** are more difficult to understand. In order to distinguish between these possibilities, (4,5–$^{13}C_2$)-**10** was synthesized from ethyl (1,2–$^{13}C_2$)-acetoacetate by a known procedure (Scheme S1)[20] and fed to *Trichoderma* sp. 34; this led to the incorporation of labeling into eight carbons of **4** with 51 % incorporation rate, resulting in two contiguous spin systems: C10–11 ($^{1}J_{C,C}$ = 43.0 Hz) and C3–2–15–14–6–7, with doublet signals for C7 and C3 ($^{1}J_{C7,C6}$ = 35.0 Hz, $^{1}J_{C3,C2}$ = 34.0 Hz) and doublets of doublets for the carbons at the internal positions (Figure 3 A, Table S1). These findings strongly support the GGPP fold implied by I-**4** for the biosynthesis of **4**, which was further supported by similar feeding experiments with (6-^{13}C)-, (2,6-$^{13}C_2$), (3-^{13}C)- and (3,5-$^{13}C_2$)-**10** (Figures 3 B–E, Table S1).

A plausible cyclization mechanism from GGPP (**13**) that is in line with all feeding experiments with the ^{13}C-labeled mevalonolactone isotopomers is illustrated in Scheme 2 B. After initial abstraction of the pyrophosphate group in **13**, the allylic cation is attacked by the terminal double bond to build a 14-membered ring system under formation of tertiary cation **A**, which is subsequently attacked by the neighboring double bond to form cation **B**. Intermediate **B** then undergoes a 1,2-hydride shift to **C**, followed by two cyclization steps to yield harzianyl cation **D**. The formation of known **7** can be explained by a terminating attack of water on **D**, whereas its deprotonation might yield **14**, which is a yet unknown diterpene. Subsequent allylic oxidation at C11 gives harzianone **4**, possibly through alcohol intermediate **8**. The other harzianone derivatives (**5**, **6**, and **9**) can be explained by additional oxidation of **4**.

The stereochemical course of the cyclization mechanism for GGPP to **4** in terms of the fate of the stereochemically distinct

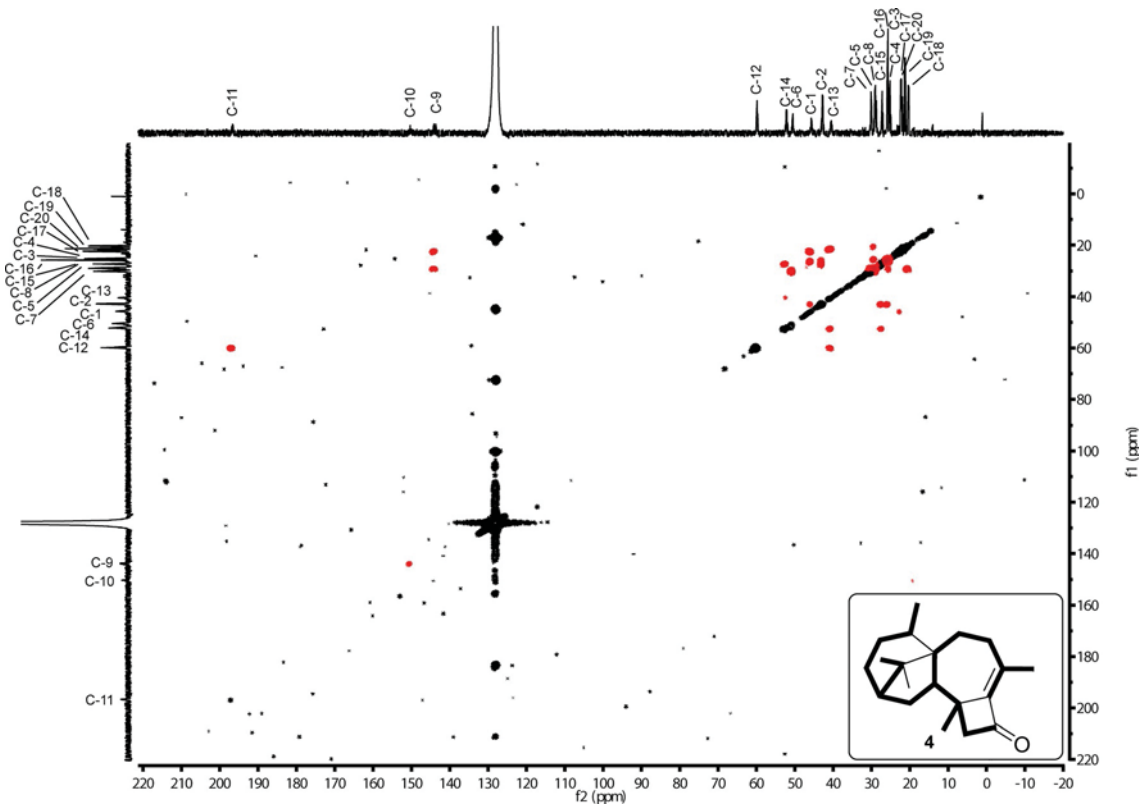
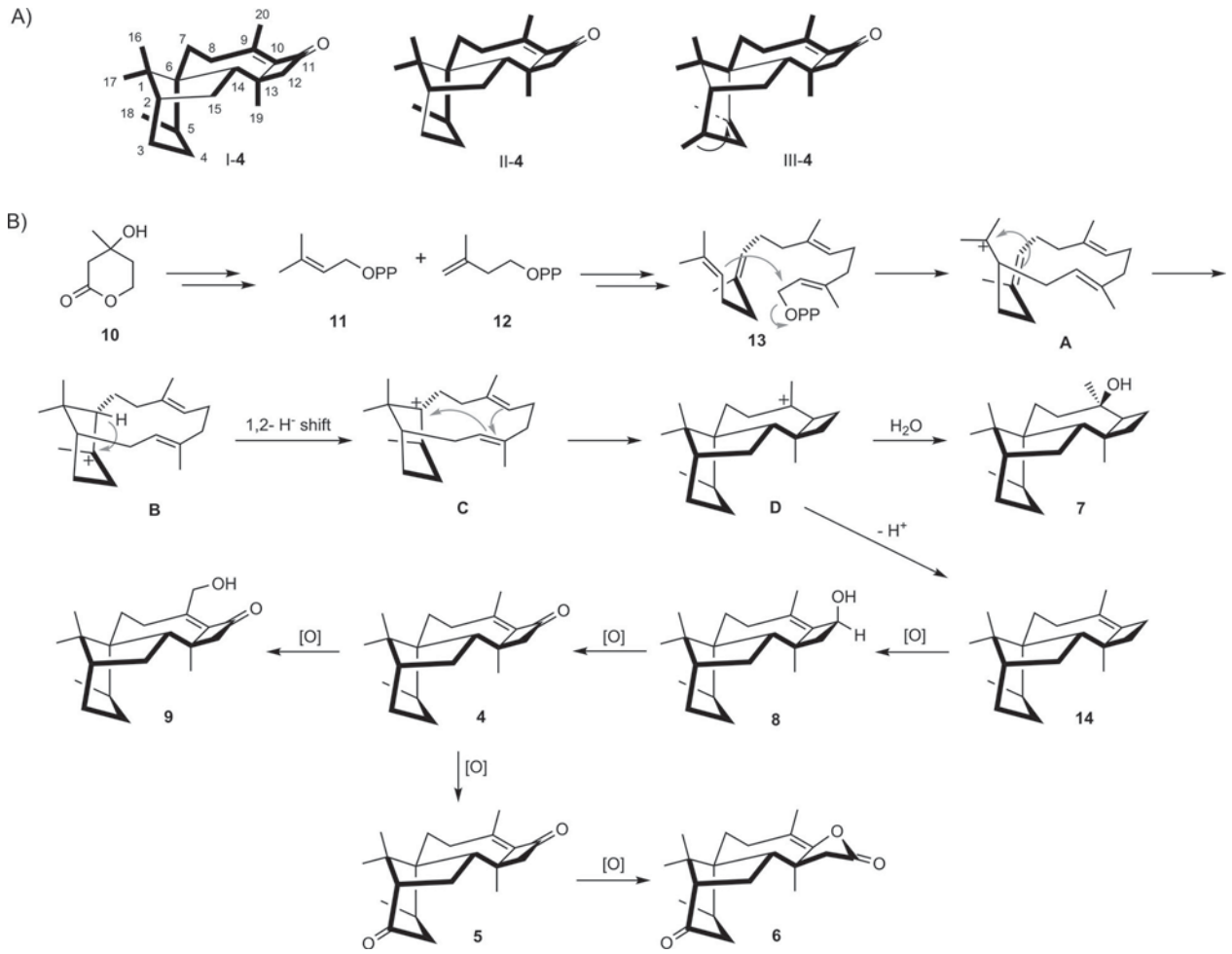


Figure 2. $^{13}C,^{13}C$ COSY spectrum of [$^{13}C_{20}$]-**4** with C_6D_6 and C,C connectivities deduced from the cross peaks shown in red (bold lines in structure).

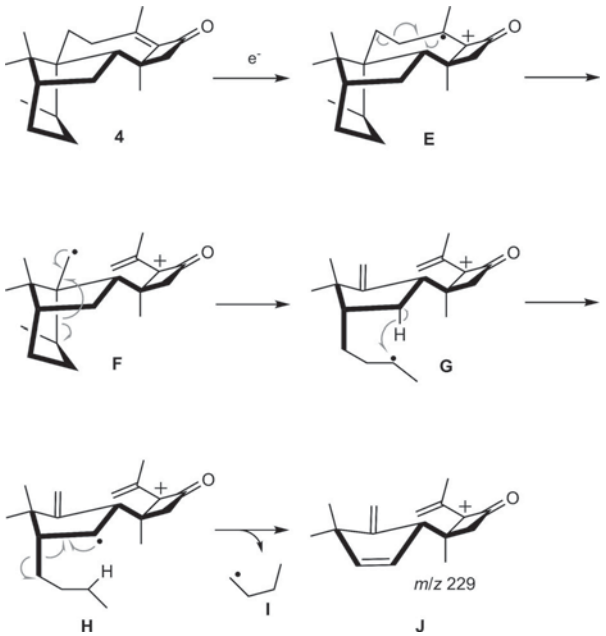
terminal geminal methyl groups could be followed by the feeding experiment with (6-^{13}C)-**10** that resulted in the specific incorporation of labeling into only one of the geminal methyl groups in **4** (C16, no incorporation into C17, Figure 3B). This finding suggested that the conformation of intermediate cation **A** was strictly controlled by the enzyme, allowing no rotation of the C1–16–17 group prior to further cyclization to **B**, and was in line with similar results obtained for various other terpenes, including 2-methylisoborneol, hypodoratoxide, and pentalenolactone.[18e,f,21] Scrambling of labeling has so far only been observed in combination with 1,2-hydride shifts into an isopropyl group, as in guaia-6,10(14)-diene and β-pinacene biosynthesis.[22]

The biosynthesis of **4** was further studied by feeding of deuterated mevalonolactones[23] and analysis of the incorporation and fragment ions by GC/MS. The advantage of using deuterated precursors was the possible gas chromatographic separation of the obtained isotopologues of **4** due to their deuterium content (Figure S3). This allowed the interpretation of the mass spectra of the maximum deuterated isotopologues that were not overlapped with mass spectra of isotopologues with a lower deuterium content that might have arisen from dilution of the fed labeled material with mevalonolactone synthesized by the fungus. The aim of the feeding experiments with the deuterated mevalonolactones was to verify the 1,2-hydride shift from **B** to **C** during the cyclization of GGPP to **14** as the precursor to **4**. This required us to localize the positions of incorporation of deuterium labeling from the EI mass spectra of deuterated **4**. For this purpose, an EI-MS fragmentation mechanism explaining the formation of the fragment ion at *m/z* 229 was developed (Scheme 3). The electron impact ionization of **4** can result in radical cation **E**, which can undergo two subsequent α-cleavage reactions via **F** to **G**. A hydrogen atom transfer results in **H**, which produces cation **J** (*m/z* 229) in another α-fragmentation with extrusion of a C_4H_9 radical (**I**).

This hypothetical fragmentation mechanism was supported by the results from three feeding experiments. First, feeding of (6,6,6-$^2H_3$)-**10** resulted in the incorporation of up to twelve deuterium atoms into **4**, as indicated by a molecular ion with *m/z* 298. Fragment ion **J** was increased by 9 amu (*m/z* 238, Figure 4A); this supports the suggested mechanism for its formation. Similarly, the feeding experiment with (2,2,6,6,6-$^2H_5$)-**10** resulted in an increase in the molecular ion of deuterated **4** to *m/z* 306 (Figure 4B), showing the incorporation of up to 20 deuterium atoms, 15 of which ended up in **J** (increased to *m/z* 244); this was again in line with the mechanism of Scheme 3. Finally, feeding of (5,5,6,6,6-$^2H_5$)-**10** produced deuterated **4** with a maximum deuterium content of eighteen deuterium atoms (*m/z* 304, Figure 4C; two deuterium atoms were lost in the oxidation of **14** to **4**). Along the fragmentation pathway to **J**, a hydrogen atom is suggested to be transferred from radical cation **G** to **H**, and this hydrogen atom was exchanged by deuterium in the feeding experiment with (5,5,6,6,6-$^2H_5$)-**10**. As a consequence, fragment ion **J** was observed at *m/z* 241. Together, all three feeding experiments supported the fragmentation mechanism for **J** shown in Scheme 3.



Scheme 2. Biosynthetic considerations for the generation of **4**. A) Different possibilities for GGPP folds (bold) to explain the formation of the harzianone skeleton. The arrow in III-**4** indicates a required carbon backbone rearrangement. B) Biosynthetic mechanism to obtain **4** and related derivatives.



Scheme 3. EI-MS fragmentation mechanism to fragment ion *m/z* 229.

The critical feeding experiment to follow the 1,2-hydride shift from intermediate **B** to **C** in the cyclization of GGPP to **4** was subsequently performed with (4,4,6,6,6-$^{2}H_5$)-**10**. The expected labeling pattern of **4** with the maximum deuterium content is shown in Figure 4D. Notably, the 1,2-hydride shift should cause incorporation of labeling at C5 of **4**. The molecular ion of deuterated **4** was observed at *m/z* 301, indicating the incorporation of fifteen of the sixteen deuterium atoms from GGPP into **4** (one deuterium atom was lost by the final deprotonation step from **D** to **14**). The fragment ion *m/z* 240 showed incorporation of eleven of these deuterium atoms into **J**; in other words, four deuterium atoms were extruded with fragment **I**. These findings are in line with the 1,2-hydride transfer from **B** to **C**.

Conclusion

In conclusion, we analyzed the cyclization mechanism of the unique diterpene harzianone (**4**) by feeding experiments using a series of isotopically labeled mevalonolactones. The results revealed a concise mechanism, resulting in a thus far unknown diterpene, which was subsequently oxidized, presumably by a

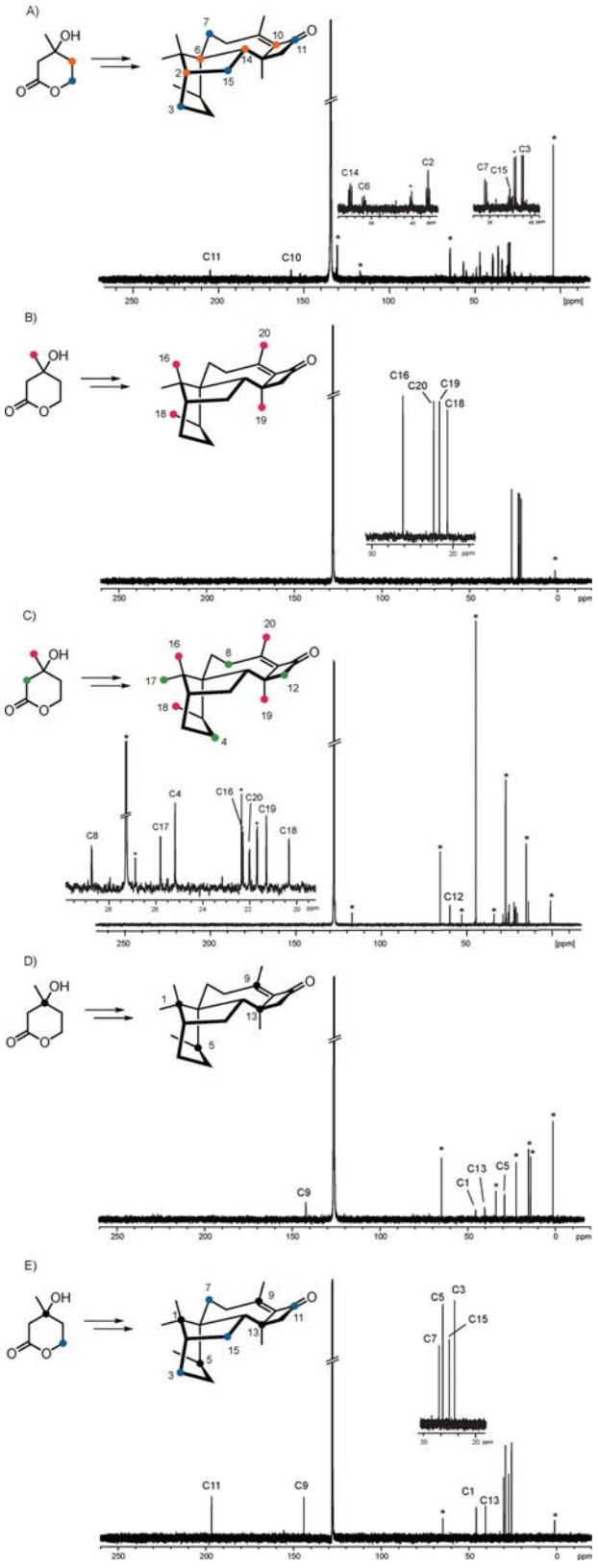


Figure 3. Results of feeding experiments. ^{13}C NMR spectra of CLSA extracts after feeding of A) (4,5-$^{13}C_2$)-**10** (51 % incorporation), B) (6-^{13}C)-**10** (91 % incorporation), C) (2,6-$^{13}C_2$)-**10** (76 % incorporation), D) (3-^{13}C)-**10** (70 % incorporation), and E) (3,5-$^{13}C_2$)-**10** (93 % incorporation). Colored dots indicate ^{13}C label. Asterisks indicate signals from solvent contaminations or other terpenoid signals.

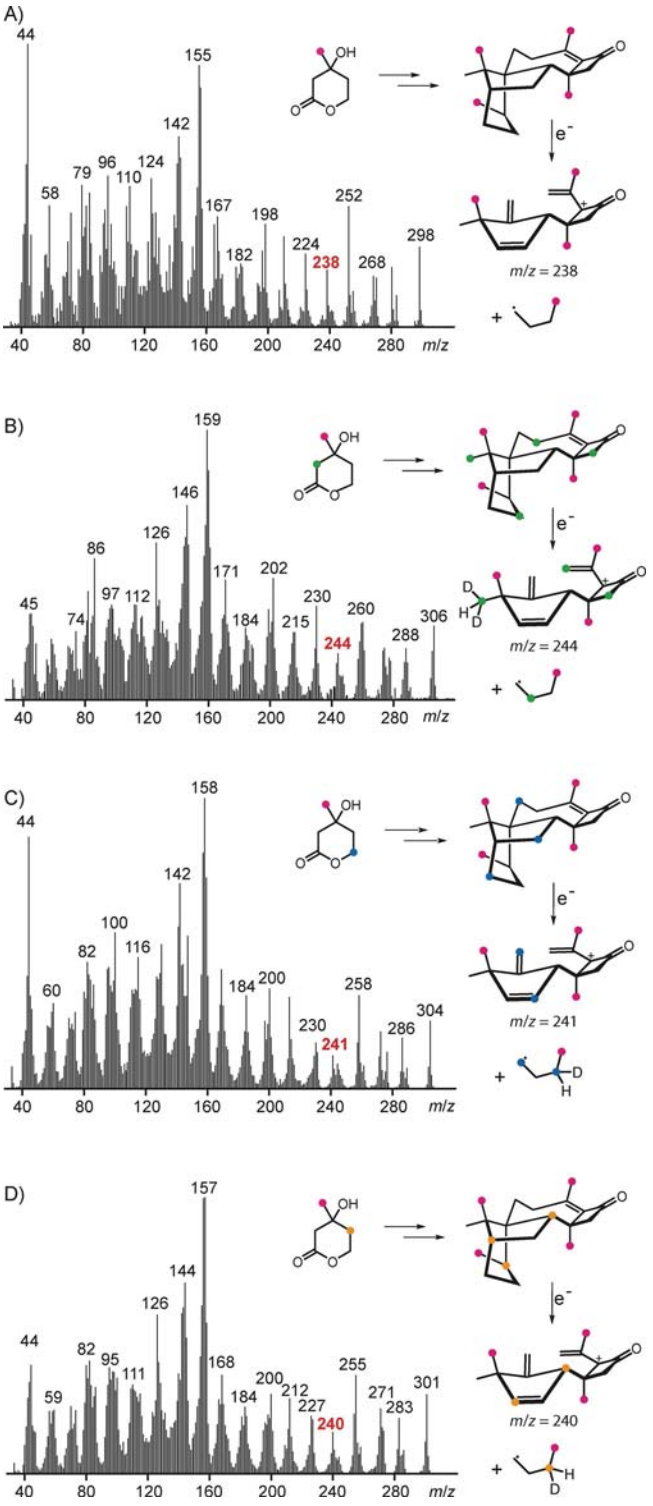


Figure 4. Results of feeding experiments with deuterated mevalonolactones. Mass spectrum of harzianone with highest deuterium incorporation after feeding of A) (6,6,6-$^2H_3$)-**10**, B) (2,2,6,6,6-$^2H_5$)-**10**, C) (5,5,6,6,6-$^2H_5$)-**10**, and D) (4,4,6,6,6-$^2H_5$)-**10**. Colored dots indicate positions of ^2H labeling.

P450 monooxygenase, to **4** and related known terpenoids. Selective labeling of the *Z*-methyl group in geranylgeranyl diphosphate by feeding of (6-^{13}C)-**10** revealed a strict stereochemical course for the first cyclization. The 1,2 hydride shift

was monitored by selective labeling and careful analysis of EI mass spectrometric data. The early steps in harzianone cyclization are identical to taxadiene biosynthesis;[24] this could point to a shared evolutionary background of the respective terpene synthases. In this context, it is interesting to note that *Trichoderma* symbionts isolated from *Taxus baccata* reportedly produce harziane diterpenes.[16] Detection of **4** in all seven analyzed *Trichoderma* strains investigated in this study indicates a conserved genetic occurrence of a harziane terpene synthase within this genus. Identification of the respective genes, followed by heterologous expression and in vitro testing, are part of our ongoing investigations.

Experimental Section

Strains and growth conditions: *Trichoderma* sp. 34, *T. asperellum* 328, *T. citrinoviride* 596, *T. harzianum* 714, *T. longibrachiatum* 594, and *T. viride* 54 were obtained from Dr. Gabriele König (University of Bonn, Germany). *T. reesei* QM 6a was obtained from the U.S. Department of Agriculture (USDA). *T. asperellum*, *T. citrinoviride*, *T. reesei*, and *T. viride* were cultivated at 28 °C in BM liquid medium (20 g malt extract per liter of deionized water), and *Trichoderma* sp. 34, *T. harzianum* 714, and *T. longibrachiatum* were cultivated at 28 °C in BM-ASW liquid medium (20 g L^{-1} malt extract, artificial sea water: 23.5 g NaCl, 10.6 g $MgCl_2{\cdot}6H_2O$, 3.92 g Na_2SO_4, 1.47 g $CaCl_2{\cdot}2H_2O$, 0.66 g KCl, 0.19 g $NaHCO_3$, 0.10 g KBr, 0.04 g $SrCl_2{\cdot}6H_2O$, 0.03 g H_3BO_3) for 10–14 days, then inoculated on agar plates of BM or BM-ASW medium. The cultures were grown for 5 to 10 days and then analyzed by CLSA.

Collection of volatiles: Emitted volatiles were collected by use of a closed-loop stripping apparatus (CLSA, Chromtech GmbH, Idstein, Precision Charcoal Filter 5 mg). Sampling was conducted for 16–20 h, and the adsorbed volatiles were eluted by extraction with analytically pure CH_2Cl_2 (40–50 μL).

Feeding experiments: Feeding experiments were performed with *Trichoderma* sp. 34 and synthetic isotopically labeled mevalonolactones (10 mg) were directly added to the medium for agar plate cultures (ca. 30 mL). *Trichoderma* sp. 34 was inoculated on the agar plates and grown for 5–10 days, followed by CLSA sampling. For feeding experiments with (2,3,4,5,6-$^{13}C_5$)-, (3-^{13}C)-, (3,5-$^{13}C_2$)-, (4,5-$^{13}C_2$)-, (2,6-$^{13}C_2$)-, and (6-^{13}C)-**10**, the volatiles were collected for 7 days, and the charcoal filter was extracted daily with C_6D_6 (7 × 50 μL). Crude extracts were directly analyzed by NMR spectroscopy. For feeding of (6,6,6-$^2H_3$)-, (2,2,6,6,6-$^2H_5$)-, (4,4,6,6,6-$^2H_5$)-, and (5,5,6,6,6-$^2H_5$)-**10**, sampling was conducted for 16–20 h, and analytically pure CH_2Cl_2 (50 μL) was used for extraction.

GC/MS analysis: The obtained headspace extracts were analyzed by using an Agilent HP7890B gas chromatograph, fitted with an HP-5MS silica capillary column (30 m, 0.25 mm i.d., 0.50 μm film), connected to an HP5977A mass detector. The GC/MS conditions were inlet pressure: 77.1 kPa, He flow: 23.3 mL min^{-1}; injection volume: 1 μL; injection mode: splitless, valve time: 60 s; oven temperature ramp: 5 min at 50 °C increasing at 5 °C min^{-1} to 320 °C; carrier gas (He) flow rate: 1 mL min^{-1}; transfer line: 250 °C; electron energy: 70 eV. Retention indices (*I*) were determined from a homologous series of *n*-alkanes (C_8–C_{40}).

General synthetic methods: All chemicals were obtained from Acros Organics (Geel, Belgium), Sigma–Aldrich Chemie GmbH (Steinheim, Germany), or TCI Deutschland GmbH (Eschborn, Germany). Solvents were purified by distillation. Whenever necessary, reactions were carried out under inert atmosphere (argon) in vacuum-heated flasks, with dry solvents (dried according to standard protocols). Thin layer chromatography (TLC) was performed on 0.20 mm silica plates (Polygram SIL G/UV254) obtained from Macherey–Nagel (Düren, Germany). Column chromatography was performed on Merck silica gel (0.040–0.063 Mesh). NMR spectra were recorded on Bruker AV I (400 MHz), AV III HD Prodigy (500 MHz), and AV III HD Cryo (700 MHz) spectrometers and were referenced against $CDCl_3$ ($\delta = 7.26$ ppm), C_6D_6 ($\delta = 7.16$ ppm), and (D$_6$)DMSO ($\delta = 2.50$ ppm) for ^{1}H NMR and $CDCl_3$ ($\delta = 77.01$ ppm), C_6D_6 ($\delta = 128.06$ ppm), and (D$_6$)DMSO ($\delta = 39.52$ ppm) for ^{13}C NMR. GC/MS analyses were carried out with an Agilent HP7890B gas chromatograph connected to a HP5977A mass detector fitted with a HP-5MS silica capillary column (30 m, 0.25 mm i.d., 0.50 μm film). The GC-MS conditions were as follows: inlet pressure: 77.1 kPa; He flow: 23.3 mL min^{-1}; injection volume: 1 μL; injection mode: split 50:1, valve time: 60 s; oven temperature ramp: 5 min at 50 °C, increasing at 10 °C min^{-1} to 320 °C; carrier gas He flow rate: 1 mL min^{-1}; transfer line: 250 °C; electron energy: 70 eV. Retention indices (*I*) were determined from a homologous series of *n*-alkanes (C_8–C_{40}). Optical rotary powers were recorded on a P8000 Polarimeter (Krüss, Hamburg, Germany).

Synthetic procedures

Synthesis of ethyl (1,2–$^{13}C_2$)-2-(2,5,5-trimethyl-1,3-dioxan-2-yl)-acetate (S1): Ethyl (1,2–$^{13}C_2$)acetoacetate (> 99 % ^{13}C, 1.00 g, 7.68 mmol, 1.0 equiv) and neopentyl glycol (1.76 g, 16.9 mmol, 2.2 equiv) were dissolved in dry CH_2Cl_2 (35 mL), and freshly distilled TMSCl (3.67 g, 33.8 mmol, 4.4 equiv) was added. The reaction mixture was heated at reflux overnight and neutralized with an aqueous solution of $NaHCO_3$ (5 wt %), followed by extraction with CH_2Cl_2 (3 × 10 mL). The organic layers were combined and dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/hexane 1:10, *v/v*, $R_f = 0.2$) to yield **S1** as a colorless oil (yield: 1.64 g, 7.50 mmol, 98 %). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.15$ (dq, $^3J_{H,H} = 7.1$ Hz, $^3J_{C,H} = 3.1$ Hz, 2 H; CH_2), 3.56 (d, $^2J_{H,H} = 11.4$ Hz, 2 H; C*H*H), 3.49 (d, $^2J_{H,H} = 11.4$ Hz, 2 H; C*H*H), 2.78 (dd, $^1J_{C,H} = 130.3$ Hz, $^2J_{C,H} = 6.9$ Hz, 2 H; CH_2), 1.53 (d, $^3J_{C,H} = 2.9$ Hz, 3 H; CH_3), 1.26 (t, $^3J_{H,H} = 7.1$ Hz, 3 H; CH_3), 0.97 (s, 3 H; CH_3), 0.94 ppm (s, 3 H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.7$ (d, $^1J_{C,C} = 58.9$ Hz, C_q), 97.3 (d, $^1J_{C,C} = 44.4$ Hz, C_q), 70.6 (d, $^3J_{C,C} = 1.6$ Hz, 2 × CH_2), 60.7 (d, $^2J_{C,C} = 1.9$ Hz, CH_2), 41.7 (d, $^1J_{C,C} = 58.9$ Hz, CH_2), 29.9 (s, C_q), 22.8 (d, $^2J_{C,C} = 4.0$ Hz, CH_3), 22.6 (s, CH_3), 22.5 (s, CH_3), 14.2 ppm (d, $^3J_{C,C} = 2.0$ Hz, CH_3); GC (BPX-5): $I = 1349$; EI-MS (70 eV): *m/z* (%) = 203 (36), 133 (29), 129 (100), 117 (18), 105 (12), 87 (31), 69 (54), 56 (36), 43 (98), 41 (49).

Synthesis of (1,2–$^{13}C_2$)-2-(2-(benzyloxy)ethyl)-2,5,5-trimethyl-1,3-dioxane (S2): $LiAlH_4$ (0.29 g, 7.50 mmol, 1.0 equiv) was suspended in dry THF (5 mL) and cooled to 0 °C. A solution of **S1** (1.64 g, 7.50 mmol, 1.0 equiv) in dry THF (5 mL) was added, and the reaction mixture was stirred at room temperature for 2 h. Water was then added until a white suspension formed. The mixture was filtered, and the obtained organic phase was dried with $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (EtOAc/hexane 1:2, *v/v*, $R_f = 0.2$). The resulting alcohol was used immediately in the next step.

The alcohol (1.13 g, 6.40 mmol, 1.0 equiv) was added dropwise at 0 °C to a suspension of NaH (0.17 g, 7.04 mmol, 1.1 equiv) in dry DMF (15 mL). After 15 min of stirring, benzyl bromide (1.09 g, 6.40 mmol, 1.0 equiv) was added dropwise, and the reaction mixture was stirred at room temperature overnight. After addition of water, the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The

organic layers were combined and washed with water and dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1:10, *v/v*, $R_f = 0.3$) to yield product **S2** (1.40 g, 5.10 mmol, 69% over 2 steps) as a colorless oil. ^{1}H NMR (400 MHz, (D_6)DMSO): $\delta = 7.36–7.23$ (m, 5H; C_6H_5), 4.43 (d, $^3J_{C,H} = 3.9$ Hz, 2H; CH_2), 3.53 (ddt, $^1J_{C,H} = 141.5$ Hz, $^3J_{H,H} = 7.3$ Hz, $^2J_{C,H} = 2.9$ Hz, 2H; CH_2), 3.46 (d, $^2J_{H,H} = 11.3$ Hz, 2H; C*H*H), 3.34 (d, $^2J_{H,H} = 11.3$ Hz, 2H; C*H*H), 1.94 (ddt, $^1J_{C,H} = 126.7$ Hz, $^3J_{H,H} = 6.7$ Hz, $^2J_{C,H} = 6.7$ Hz, 2H; CH_2), 1.30 (d, $^3J_{C,H} = 2.9$ Hz, 3H; CH_3), 0.90 (s, 3H; CH_3), 0.81 ppm (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 138.6$ (d, $^3J_{C,C} = 3.0$ Hz, C_q), 128.2 (s, 2×CH), 127.4 (s, 2×CH), 127.3 (s, CH), 97.5 (d, $^1J_{C,C} = 46.2$ Hz, C_q), 71.9 (dd, $^2J_{C,C} = 3.6$ Hz, $^3J_{C,C} = 1.4$ Hz, CH_2), 69.2 (d, $^3J_{C,C} = 1.8$ Hz, 2×CH_2), 65.6 (d, $^1J_{C,C} = 39.3$ Hz, *C*H_2), 37.9 (d, $^1J_{C,C} = 39.3$ Hz, *C*H_2), 29.5 (s, C_q), 22.4 (s, CH_3), 22.1 (s, CH_3), 20.9 ppm (d, $^3J_{C,C} = 3.3$ Hz, CH_3); GC (BPX-5): $I = 1878$; EI-MS (70 eV): *m/z* (%) = 251 (26), 179 (2), 162 (7), 129 (78), 107 (29), 91 (100), 69 (45), 56 (29), 43 (66), 41 (45).

Synthesis of (3,4–$^{13}C_2$)-4-(benzyloxy)butan-2-one (S3): Compound **S2** (1.40 g, 5.10 mmol, 1 equiv) was dissolved in MeOH (25 mL), and a solution of HCl in water (1.0 M, 3 mL) was added. The reaction mixture was stirred at room temperature for 15 min, then neutralized with an aqueous solution of $NaHCO_3$ (5 wt%). The aqueous phase was extracted with Et_2O (3×10 mL), and the combined organic layers were dried with $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (EtOAc/hexane 1:5, *v/v*, $R_f = 0.2$) to yield product **S3** (0.90 g, 5.00 mmol, 99%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.37–7.25$ (m, 5H; C_6H_5), 4.51 (d, $^3J_{C,H} = 4.3$ Hz, 2H; CH_2), 3.74 (ddt, $^1J_{C,H} = 143.2$ Hz, $^3J_{H,H} = 6.3$ Hz, $^2J_{C,H} = 2.8$ Hz, 2H; CH_2), 2.72 (ddt, $^1J_{C,H} = 126.0$ Hz, $^3J_{H,H} = 6.3$ Hz, $^2J_{C,H} = 4.9$ Hz, 2H; CH_2), 2.18 ppm (d, $^3J_{C,H} = 1.3$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 207.1$ (dd, $^1J_{C,C} = 40.6$ Hz, $^2J_{C,C} = 1.7$ Hz, C_q), 138.1 (d, $^3J_{C,C} = 2.9$ Hz, C_q), 128.4 (s, 2×CH), 127.7 (s, 2×CH), 127.6 (s, CH), 73.2 (dd, $^2J_{C,C} = 3.9$ Hz, $^3J_{C,C} = 1.4$ Hz, CH_2), 65.3 (d, $^1J_{C,C} = 39.8$ Hz, CH_2), 43.7 (d, $^1J_{C,C} = 39.8$ Hz, CH_2), 30.4 ppm (d, $^2J_{C,C} = 14.2$ Hz, CH_3); GC (BPX-5): $I = 1467$; EI-MS (70 eV): *m/z* (%) = 121 (21), 107 (59), 91 (100), 79 (39), 77 (37), 65 (38), 59 (12), 43 (90).

Synthesis of ethyl (4,5–$^{13}C_2$)-5-(benzyloxy)-3-hydroxy-3-methylpentanoate (S4): Diisopropylamine (1.06 g, 10.5 mmol, 2.1 equiv) was dissolved in dry THF (50 mL) and a solution of *n*-butyllithium in hexane (1.6 M, 6.56 mL, 10.5 mmol, 2.1 equiv) was added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and then cooled to −78 °C. A solution of EtOAc (0.93 g, 10.5 mmol, 2.1 equiv) in dry THF (20 mL) was added dropwise, and the reaction mixture was stirred for 30 min. A solution of **S3** (0.90 g, 5.00 mmol, 1.0 equiv) in dry THF (15 mL) was added dropwise, and the mixture was stirred for 1 h, quenched by addition of water, and allowed to warm to room temperature. The reaction mixture was extracted with EtOAc (3×15 mL), and the combined organic layers were dried with $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel (EtOAc/hexane 1:5, *v/v*, $R_f = 0.2$) to yield product **S4** (1.10 g, 4.10 mmol, 82%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.33–7.17$ (m, 5H; C_6H_5), 4.43 (d, $^3J_{C,H} = 4.0$ Hz, 2H; CH_2), 4.11–4.02 (m, 2H CH_2), 3.90 (d, $^3J_{C,H} = 2.4$ Hz, 1H; OH), 3.62 (ddt, $^1J_{C,H} = 141.9$ Hz, $^3J_{H,H} = 6.2$ Hz, $^2J_{C,H} = 2.8$ Hz, 2H; CH_2), 2.52 (dd, $^2J_{H,H} = 15.2$ Hz, $^3J_{C,H} = 2.9$ Hz, 1H; C*H*H), 2.43 (dd, $^2J_{H,H} = 15.2$ Hz, $^3J_{C,H} = 2.9$ Hz 1H; C*H*H), 1.85 (ddt, $^1J_{C,H} = 126.3$ Hz, $^3J_{H,H} = 6.2$ Hz, $^2J_{C,H} = 4.6$ Hz, 2H; CH_2), 1.21 (d, $^3J_{C,H} = 3.7$ Hz, 3H; CH_3), 1.18 ppm (t, $^3J_{H,H} = 7.1$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.6$ (d, $^3J_{C,C} = 2.3$ Hz, C_q), 138.1 (d, $^3J_{C,C} = 2.6$ Hz, C_q), 128.5 (s, 2×CH), 127.8 (s, CH), 127.8 (s, 2×CH), 73.4 (dd, $^2J_{C,C} = 3.9$ Hz, $^3J_{C,C} = 1.4$ Hz, CH_2), 70.9 (dd, $^1J_{C,C} = 38.5$ Hz, $^2J_{C,C} = 1.33$ Hz, C_q), 67.1 (d, $^1J_{C,H} = 38.8$ Hz, CH_2), 60.6 (s, CH_2), 45.5 (dd, $^2J_{C,C} = 1.9$ Hz, $^3J_{C,C} = 1.9$ Hz, CH_2), 40.3 (d, $^1J_{C,C} = 38.5$ Hz, CH_2), 27.1 (dd, $^2J_{C,C} = 1.9$ Hz, $^3J_{C,C} = 1.9$ Hz, CH_3), 14.1 ppm (s, CH_3); GC (BPX-5) = 1916; EI-MS (70 eV): *m/z* (%) = 181 (1), 162 (21), 144 (13), 132 (8), 113 (7), 91 (100), 65 (20), 43 (38).

Synthesis of (4,5–$^{13}C_2$)mevalonolactone ((4,5-$^{13}C_2$)-10): Compound **S4** (1.10 g, 4.10 mmol, 1 equiv) was dissolved in MeOH (40 mL), and Pd/C (5 wt%, 0.05 equiv) was added. The mixture was stirred at 40 °C under a hydrogen atmosphere (40 bar) for 2 h. The Pd/C catalyst was removed by filtration over celite, and the organic phase was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (40 mL), and catalytic amounts of *p*-TsOH were added. The reaction mixture was stirred at room temperature overnight, followed by removal of the solvent under reduced pressure and purification of the residue by column chromatography (EtOAc/hexane, 1:1 *v/v*, $R_f = 0.1$) to yield (4,5-$^{13}C_2$)-**10** (0.33 g, 2.50 mmol, 62%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.53$ (dm, $^1J_{C,H} = 150.4$ Hz, 1H; C*H*H), 4.32 (dm, $^1J_{C,H} = 150.4$ Hz, 1H; C*H*H), 2.65 (ddd, $^2J_{H,H} = 17.4$ Hz, $^3J_{C,H} = 3.5$ Hz, $^4J_{H,H} = 1.7$ Hz, 1H; C*H*H), 2.53 (dd, $^2J_{H,H} = 17.4$ Hz, $^3J_{C,H} = 1.5$ Hz, 1H; CH*H*), 1.87 (dm, $^1J_{C,H} = 130.6$ Hz, 2H; CH_2), 1.32 ppm (d, $^3J_{C,H} = 4.2$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$ (s, C_q), 68.3 (dd, $^1J_{C,C} = 37.1$ Hz, $^2J_{C,C} = 2.3$ Hz, C_q), 66.2 (d, $^1J_{C,C} = 34.9$ Hz, CH_2), 44.5 (d, $^2J_{C,C} = 1.7$ Hz, CH_2), 35.0 (d, $^1J_{C,C} = 34.9$ Hz, CH_2), 29.9 ppm (dd, $^2J_{C,C} = 1.9$ Hz, $^3J_{C,C} = 1.9$ Hz, CH_3; GC (BPX-5, MSTFA): $I = 1390$; EI-MS (70 eV, MSTFA): *m/z* (%) = 189 (12), 147 (100), 145 (39), 117 (47), 116 (48), 75 (51), 73 (45), 45 (24).

Isolation of harzianone (4): *Trichoderma* sp. 34 was precultured at 28 °C in liquid BM-ASW medium for 10–14 days, then inoculated on 100 agar plates in BM-ASW medium (3 L). The cultures were grown for 21 days, cut into small pieces, and extracted with pentane. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (diethyl ether/pentane 20:1→5:1, *v/v*, $R_f = 0.3$) to yield harzianone (**4**) (3.8 mg, 0.01 mmol): $[\alpha]_{21}^{D} = +21.0$ (*c* 0.1, MeOH); ^{1}H NMR (700 MHz, C_6D_6): $\delta = 2.33$ (d, $^2J = 15.8$ Hz, 1H; C*H*H), 2.24 (d, $^2J = 15.8$ Hz, 1H; C*H*H), 2.20 (dd, $^3J = 7.8$ Hz, $^2J = 7.8$ Hz, 1H; CH), 2.06 (s, 3H; CH_3), 2.04 (m, 1H; C*H*H), 1.97 (dd, $^3J = 11.4$ Hz, $^3J = 8.9$ Hz, 1H; CH), 1.92 (m, 1H; C*H*H), 1.86 (m, 1H; C*H*H), 1.60 (m, 1H; C*H*H), 1.51 (m, 1H; CH), 1.49 (m, 1H; C*H*H), 1.42 (m, 1H; C*H*H), 1.24 (s, 3H; CH_3), 1.21 (m, 1H; C*H*H), 1.12 (m, 1H; C*H*H), 1.11 (m, 1H; C*H*H), 1.03 (m, 1H; C*H*H), 0.94 (s, 3H; CH_3), 0.86 (d, $^3J = 7.5$ Hz, 3H; CH_3), 0.70 ppm (s, 3H; CH_3); ^{13}C NMR (175 MHz, C_6D_6): $\delta = 197.0$ (C_q), 150.8 (C_q), 144.3 (C_q), 60.2 (CH_2), 52.6 (CH), 50.9 (C_q), 46.2 (C_q), 43.2 (CH), 40.9 (C_q), 30.5 (CH_2), 29.5 (CH), 29.1 (CH_2), 27.6 (CH_2), 26.2 (CH_3), 25.7 (CH_2), 25.6 (CH_2), 22.7 (CH_3), 22.4 (CH_3), 21.7 (CH_3), 20.7 ppm (CH_3); GC (HP-5MS) = 2280; EI-MS (70 eV): *m/z* (%) = 286 (23), 271 (34), 257 (40), 243 (60), 229 (30), 215 (40), 202 (46), 189 (51), 173 (34), 159 (57), 149 (100), 136 (66), 121 (89), 105 (71), 91 (94), 79 (89), 55 (60), 41 (86).

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Conflict of Interest

The authors declare no conflict of interest.

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