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Genetic Dissection of Sesquiterpene Biosynthesis by *Fusarium fujikuroi*

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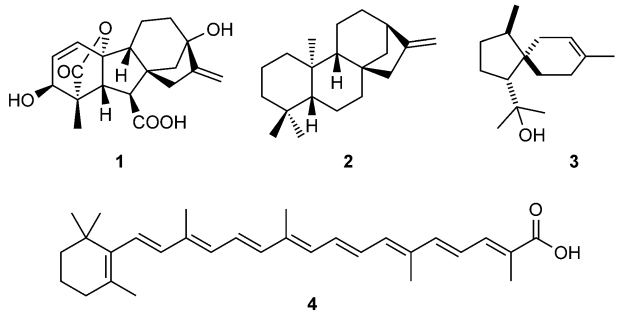
The rice pathogenic ascomycete *Fusarium fujikuroi* IMI58289 is a well-known producer of secondary metabolites of various compound classes. Its biosynthetic potential is reflected by the production of the polyketides bikaverin,[1] fusarubins,[2] and fumonisins,[3] the mixed polyketide/non-ribosomal peptide fusarin C,[4] and fusaric acid and moliniformin of unknown biosynthetic pathways.[5,6] Terpenoids include the phytohormone gibberellic acid (GA_3, **1**) and several of its derivatives (Scheme 1).[7]



Scheme 1. Known terpenoids from *F. fujikuroi*.

In plants and associated rhizobacteria the gibberellins are made in small amounts and act as beneficial plant growth promoters.[8,9] Elevated rates of gibberellin biosynthesis by *F. fujikuroi* induce plant overgrowth, thereby resulting in "foolish seedling" disease (bakanae), which is responsible for severe crop damage worldwide. Gibberellin biogenesis starts with the formation of *ent*-kaurene (**2**) by the bifunctional *ent*-copalyl diphosphate synthase/*ent*-kaurene synthase (CPS/KS).[10] Further terpenes from *F. fujikuroi* are the spirocyclic sesquiterpene α-acorenol (**3**)[11] and the carotenoid neurosporaxanthin (**4**), which arises from a branch of the β-carotene biosynthetic pathway.[12,13]

Fungal sesquiterpene biosynthesis starts from the well-established mevalonate pathway, in which three molecules of acetyl-CoA give the activated C_5-units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Their conjunction product farnesyl diphosphate (C_{15}) harbors one moiety of DMAPP and two units of IPP and is the linear precursor for all sesquiterpenoids. Cascade reactions via cationic intermediates are catalyzed by terpene cyclases; the simple achiral molecule FPP can be converted in one step into highly complex, often polycyclic, natural products that can contain several defined stereocenters. These cascades terminate by simple deprotonation to give sesquiterpene hydrocarbons, or by attack of water to give the corresponding sesquiterpenoid alcohols.[14]

We have recently investigated the volatiles of *F. fujikuroi* IMI58289 by use of a closed-loop stripping apparatus (CLSA) in combination with GC-MS.[11] A representative chromatogram of headspace extract is shown in Figure 1 A. Besides **2**, several structurally related diterpenes were identified as side-products of the CPS/KS, by knockout experiments. The sesquiterpenoid content could be divided in two groups based on their pro-

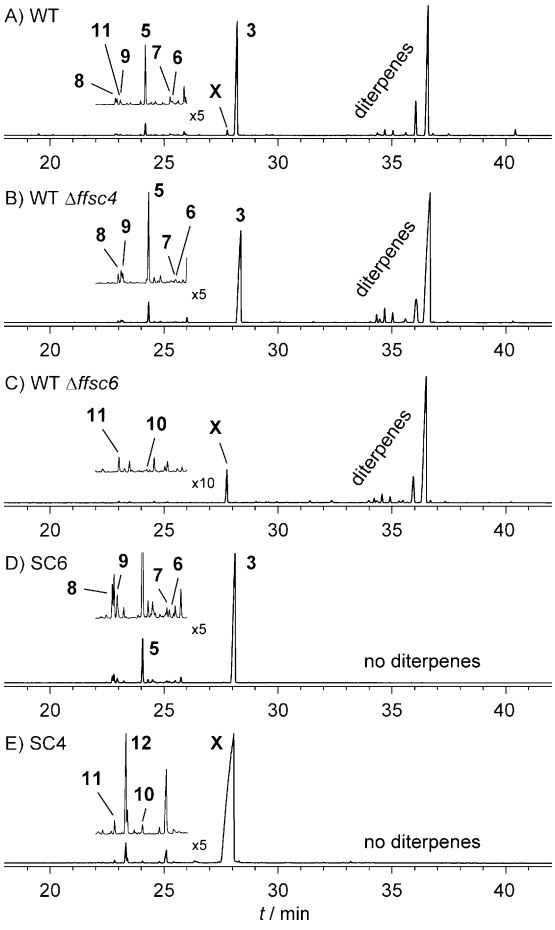


Figure 1. Total ion chromatograms of *F. fujikuroi* IMI58289 headspace extracts. A) wild-type, B) Δ*ffsc4*, C) Δ*ffsc6*, D) SG139 *gpd*::*ffsc6* Δ*ffsc4*, and E) SG139 *gpd*::*ffsc4* Δ*ffsc6*.

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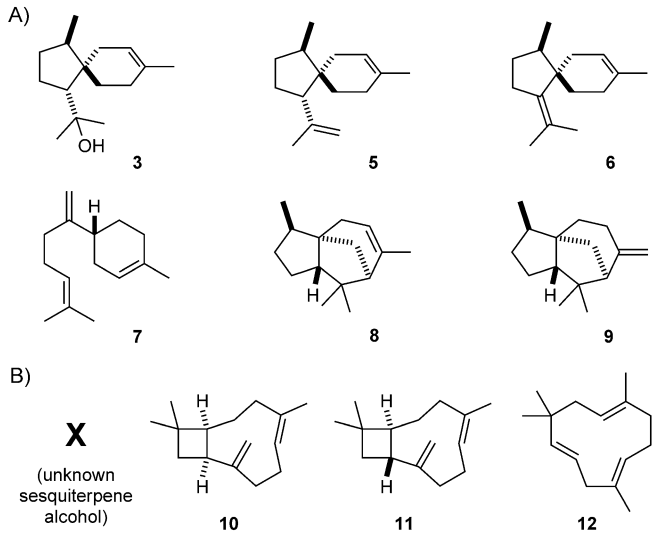
posed biosynthetic pathways (Schemes S1 and S2 in the Supporting Information). The first group is represented by the main compound (**3**) accompanied by the acorane-type sesquiterpenes α-acoradiene (**5**) and α-alaskene (**6**, Scheme 2A). Other compounds such as β-bisabolene (**7**) were suggested to



Scheme 2. Two groups of volatile sesquiterpenoids are produced by *F. fujikuroi*. A) Products of the sesquiterpene cyclase Ffsc6, B) products of Ffsc4.

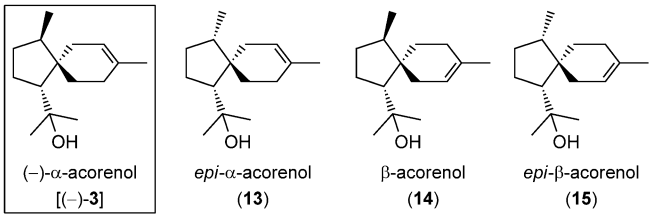
arise by deprotonations from cationic intermediates along the pathway to the acorane skeleton, whereas α-(**8**) and β-cedrene (**9**) require an additional cyclization step. The second group of sesquiterpenoids comprises an unidentified terpene alcohol (**X**; mass spectrum shown in Figure S1) along with the caryophyllanes 2-*epi*-(*E*)-β-caryophyllene (**10**) and (*E*)-β-caryophyllene (**11**, Scheme 2B). α-Humulene (**12**) was assumed to arise from the intermediate (*E*,*E*)-humulyl cation by deprotonation prior to a second cyclization step to the caryophyllane skeleton.

Although most of the characterized terpene cyclases are from plants, little is known about fungal terpene cyclases. The functionally characterized fungal enzymes include the trichodiene synthase from *Fusarium sporotrichioides* and the aristolochene synthases from *Penicillium roqueforti* and *Aspergillus terreus*, for which crystal structures have been obtained.[15–19] Recently, the presilphiperfolan-8β-ol synthase from *Botrytis cinerea* was described.[20,21] Here we present the identification of two terpene cyclases from *F. fujikuroi*, the construction of genetically engineered strains for optimized terpene production, and the isolation and structural elucidation of the enzymes' main products.

Examination of the recently sequenced genome of *F. fujikuroi* (B.T. and co-workers, unpublished results) revealed six putative sesquiterpene-cyclase-encoding genes, *ffsc1–ffsc6*. To identify their specific products, each gene was deleted, and changes in the volatile metabolome were monitored with the CLSA technique. For the Ffsc4 and Ffsc6 knockout mutants (*F. fujikuroi* sesquiterpene cyclases 4 and 6; gene loci FFUJ_12585 and FFUJ_10353) significant differences were obtained in the production of volatile sesquiterpenes, compared to wild-type. The Δ*ffsc4* mutant showed abolished production of **X** and related hydrocarbons, whereas the Δ*ffsc6* strain was impaired in the production of **3** and its side products (Figure 1B and C).

For a full structural characterization of **X** and for an assignment of the absolute configuration of **3**, purification of these sesquiterpene alcohols from *F. fujikuroi* cultures was undertaken. For this, their production was improved by genetic engineering. As a recipient strain for the combined knock-out and overexpression approach, we used the GA-deficient mutant strain SG139 (lacking the 30-kb GA biosynthesis gene cluster).[22] Headspace extracts of this strain contain a much less complex pattern of volatiles, and formation of all diterpenes is abolished in this strain.[11] This strain was selected for the overproduction of sesquiterpenes formed by Ffsc6 or Ffsc4, by complementary introduction of the *gpd*::*ffsc6* or the *gpd*::*ffsc4* overexpression vectors with concomitant deletion of the other gene. The *gpd* promoter is derived from the glycolysis enzyme glyceraldehyde-3-phosphate dehydrogenase and was induced under the growth conditions in glucose-containing medium for terpene production. The resulting strains were *F. fujikuroi* SC6 (SG139 *gpd*::*ffsc6* Δ*ffsc4*) for the production of **3** and *F. fujikuroi* SC4 (SG139 *gpd*::*ffsc4* Δ*ffsc6*) for the caryophyllane-type alcohol **X**. In both mutants, considerably increased production of the respective sesquiterpene by the terpene cyclase under the *gpd* promoter was observed (ca. threefold increase in production of **3**, Figure 1D; ca. 100-fold for **X**, Figure 1E). The strains *F. fujikuroi* SC6 and SC4 were used for isolation of the two principal components.

Extraction from 50 agar plates of *F. fujikuroi* SC6 and chromatography on silica gel yielded 15 mg of pure **3**. This sesquiterpene alcohol and its diastereomers *epi*-α-acorenol (**13**), β-acorenol (**14**), and *epi*-β-acorenol (**15**; Scheme 3) had similar mass



Scheme 3. The four diastereomers of acorenol.

spectra and retention indices. Because our previous structural assignment for **3** was based only on GC-MS data, a reassignment was performed, by comparison of the NMR data of the isolated sesquiterpene alcohol to published data for all four acorenol stereoisomers (Table 1).[23] This confirmed the previously suggested structure of α-acorenol. The absolute configuration of **3** was established from the optical rotatory power, $[\alpha]_D^{23} = -15.5$ ($c = 0.12$, $CHCl_3$), which was in good agreement with the specific rotation of synthetic (−)-α-acorenol, $[\alpha]_D^{23} = -16.5$ ($c = 1.60$, $CHCl_3$).[24]

Similarly, extraction from 50 agar plates of *F. fujikuroi* SC4 followed by silica gel chromatography yielded 8 mg of pure **X**. Its

Table 1. Selected ^{1}H NMR data for the Ffsc6 product and the four acorenol diastereomers[23]

			δ (ppm)		
	Ffsc6	**3**	**13**	**14**	**15**
14-H	0.86	0.86	0.87	0.83	0.94
13-H	1.22	1.21	1.24	1.26	1.25
12-H	1.22	1.22	1.25	1.32	1.29
7-H	5.42	5.42	5.41	5.33	5.31

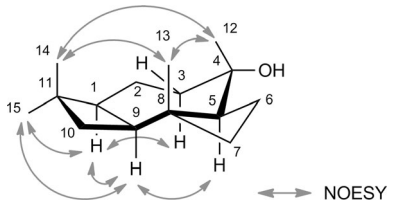


Figure 3. NOESY correlations of **X**.

structure was rigorously determined by one- and two-dimensional NMR techniques. The absence of signals for olefinic double bonds in the ^{1}H and ^{13}C NMR spectra suggested a tricyclic skeleton (sesquiterpene alcohols $C_{15}H_{26}O$ have three degrees of unsaturation). The ^{1}H NMR spectrum showed signals for four methyl groups ($\delta = 0.86$, 1.13, 1.21, and 1.25 ppm), ten methylene protons ($\delta = 1.32$–1.95 ppm), three methine protons ($\delta = 1.99$, 2.04, and 2.59 ppm), and one broad signal at $\delta = 1.63$ ppm for the alcohol proton. Accordingly, the ^{13}C NMR spectrum showed four signals for methyl groups ($\delta = 22.3$, 23.4, 24.2, and 31.0 ppm), five methylene carbons ($\delta = 18.7$, 22.2, 34.0, 37.6, and 46.3 ppm), three methine carbons ($\delta = 42.8$, 50.4, and 53.2 ppm), and three signals for quaternary carbons ($\delta = 32.3$ and 42.3 ppm, and one oxygenated carbon at 74.0 ppm). The assignments of ^{13}C NMR data were confirmed by distortionless enhancement by polarization transfer (DEPT) spectroscopy. A planar structure with a 4-7-4-skeleton for **X** was concluded from the H,H COSY and HMBC correlations (Figure 2A). NMR data have previously been published for three of the 16 possible diastereomers: 5,8-cyclocaryophyllen-4α-ol (**16**),[25] 5,8-cyclocaryophyllen-4β-ol (**17**),[25] and koraiol (**18**) (Figure 2B).[26] The published NMR data for **18** were incomplete and therefore not useful, whereas comparison of our data to those of **16** and **17** clearly excluded these candidate structures for **X**.

The relative configuration of **X** was elucidated by NOESY experiments (Figure 3). The *cis* fusion of rings A and B was deduced from the NOESY couplings of the bridgehead hydrogen

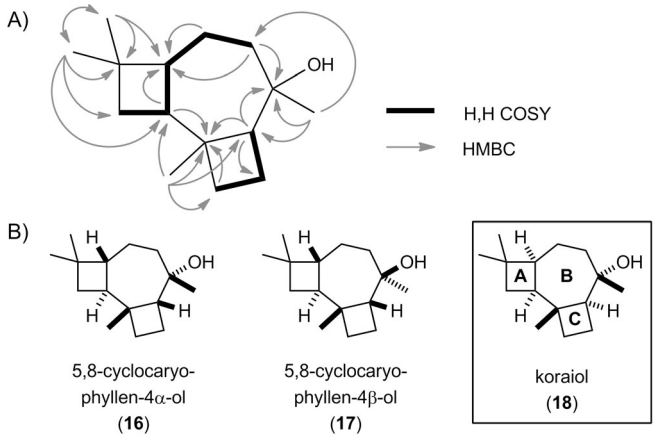


Figure 2. Structure elucidation of **X**. A) Planar structure deduced from H,H COSY and key HMBC couplings, B) structures of known stereoisomers of **X**.

atoms 1-H and 9-H with each other, with methyl protons 15-H, and the *pro-S*-methylene proton 3-H. The second A-ring methyl group (C-14) showed NOESY correlations to the methyl protons 12-H and 13-H, thus indicating that they are facing the same side of the molecule. The *trans* fusion of rings B and C became evident as 5-H did not show correlations to 12-H or 13-H, but instead to 9-H, thus establishing the identity of **X** and koraiol (**18**).

In summary, the main products of two sesquiterpene synthases from *F. fujikuroi* have been identified as (−)-α-acorenol (Ffsc6, FFUJ_10353) and koraiol (Ffsc4, FFUJ_12585) by genetically engineered production optimization, compound purification, and structure elucidation by spectroscopic methods. The knockout and overproduction mutants *F. fujikuroi* SC6 and SC4 also confirmed the correctness of our previously suggested grouping of sesquiterpenes according to their common biosynthetic origin from one terpene cyclase (Table S1). Future work will include a genome-mining approach for the identification of further silenced terpene cyclases encoded in the genome of *F. fujikuroi*.

Experimental Section

Accession numbers. The gene sequences of the terpene cyclases were deposited under accession numbers HF563560 and HF563561.

Strains and culture conditions. *F. fujikuroi* IMI58289 was precultured on CM agar medium (salt solution (50 mL L^{-1}), micronutrient solution (1 mL L^{-1}), vitamin solution (1 mL L^{-1}), yeast extract (1 g L^{-1}), peptone (2 g L^{-1}), casamino acids (1 g L^{-1}), glucose (10 g L^{-1}), agar (15 g L^{-1})) at 28 °C, where salt solution is KCl (10.4 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (10.4 g L^{-1}), KH_2PO_4 (30.4 g L^{-1}), micronutrient solution is $FeSO_4 \cdot 7H_2O$ (10 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (50 g L^{-1}), and vitamin solution is biotin (0.5 g L^{-1}), nicotinic acid (50 g L^{-1}), 4-aminobenzoic acid (PABA; 16 g L^{-1}), and pyridoxal hydrochloride (20 g L^{-1}). The agar plates with CM agar medium were inoculated with preculture (~0.25 cm^2), incubated (3–4 days, 28 °C), and directly subjected to headspace analysis.

Knockout of *ffsc4* and *ffsc6*. Plasmids pΔ*ffsc6* and pΔ*ffsc4* and overexpression constructs p*gpd*::*ffsc6* and p*gpd*::*ffsc4* were assembled by using yeast recombinatorial cloning as described for *Neurospora crassa* deletion vectors (basis vector: pCSN44) and recently established for *F. fujikuroi* vectors.[27,28] The 5′ and 3′ flanking regions of *ffsc6* and *ffsc4* were amplified by using primer pairs TC-12585-5F/5R and TC-12585-3F/3R, and TC-10353-5F/5R and TC-10353-3F/3R, respectively (for primer sequences see Table S2). For the generation of Ffsc6 and Ffsc4 overexpression vectors, primer pairs gpd-TC_12585 F/R and gpd-TC_10353_F/R, respectively, were

used. The amplicons were cloned into vector pgpd_nat, which contains the nourseothricin-resistance cassette. Plasmid DNA was extracted from yeast by using the GeneJET Plasmid Miniprep Kit (Fermentas/Thermo Scientific).

Fungal transformations. Preparation of protoplasts from *F. fujikuroi* mycelium was carried out as described.[29] For gene knockouts, 10^7 protoplasts of *F. fujikuroi* were transformed with 10 μg of the replacement cassette vectors pΔ*ffsc6* and pΔ*ffsc4*. Transformed protoplasts were regenerated for 6–7 days at 28 °C in regeneration agar (sucrose (0.7 M), yeast extract (0.05 %)) containing hygromycin (100 μg mL^{-1}). For overexpression of *ffsc6*, the Δ*ffsc4* mutant was transformed with vector p*gpd*::*ffsc6*, and for overexpression of *ffsc4* the Δ*ffsc6* mutant was transformed with vector p*gpd*::*ffsc4*. Transformed protoplasts were regenerated in regeneration agar containing nourseothricin (100 μg mL^{-1}). Homologous integration events (replacement of *ffsc6* or *ffsc4* with the hygromycin-resistance marker) were verified by PCR (primer pairs TC-10353-dia-3R or TC-12585-dia-3R in combination with pLOF-OliP, and TC-10353-dia-5F or TC-12585-dia-5F in combination with Tub-T, respectively). The absence of wild-type gene loci in deletion strains was verified by PCR (primer pairs TC-10353-WT-F/R and TC-12585-WT-F/R, targeting replaced coding region of the respective gene).

Headspace analyses. The volatiles emitted by fungal agar plate cultures were collected by use of a CLSA as described previously.[11] Headspace extracts were analyzed by GC-MS on an HP 7890A GC system connected to an HP5975C Mass Selective Detector fitted with a HP-5 MS fused silica capillary column (30 m × 0.22 mm i.d., 0.25 μm film; Agilent). Conditions were : inlet pressure, 67 kPa He (23.3 mL min^{-1}); injection volume, 1 μL; injector, 250 °C; transfer line, 300 °C; electron energy, 70 eV. The GC was programmed as follows: 50 °C (5 min isothermic), increase (5 °C min^{-1}) to 320 °C, and operated in splitless mode; carrier gas, He (1.2 mL min^{-1}). Retention indices were determined from a homologous series of *n*-alkanes (C_8–C_{32}). Compound identification was performed by comparison of mass spectra to database spectra (Table S1).

Purification of α-acorenol (3) and koraiol (18). *F. fujikuroi* SC6 (SG139 *gpd*::*ffsc6* Δ*ffsc4*, for α-acorenol) and *F. fujikuroi* SC4 (SG139 *gpd*::*ffsc4* Δ*ffsc6*, for koraiol) were precultured in CM medium at 28 °C. For each strain, 50 agar plates were inoculated with the respective preculture (1 mL), incubated (3 days, 28 °C), and extracted with ethyl acetate. The extracts were dried with $MgSO_4$, and concentrated in vacuo. Purification by column chromatography on silica gel (hexane/ethyl acetate10:1) gave **3** (15 mg) and **18** (8 mg) as colorless oils.

α-Acorenol (3). R_f = 0.25 (hexane/ethyl acetate 10:1); I = 1632; $[\alpha]_D^{23} = -15.5$ (c = 0.12, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ = 5.42 (m, 1H; CH), 2.35 (d, ^2J(H,H) = 17.8 Hz, 1H; CH_2), 2.05–1.93 (m, 3H; 2 CH_2), 1.93–1.83 (m, 3H; 3 CH_2) 1.80–1.74 (m, 1H; CH_2), 1.66 (s, 3H; CH_3), 1.56–1.50 (m, 2H; 2CH_2) 1.40 (dddd, ^2J(H,H) = 13.3 Hz, ^3J(H,H) = 7.7 Hz, ^3J(H,H) = 5.6 Hz, ^4J(H,H) = 1.0 Hz, 1H; CH_2), 1.29–1.24 (m, 1H; CH_2) 1.22 (s, 3H; CH_3), 1.22 (s, 3H; CH_3), 0.86 ppm (d, ^3J(H,H) = 5.6 Hz, 3H; CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 135.2 (C_q), 121.2 (CH), 73.8 (C_q), 54.8 (CH), 45.0 (C_q), 41.7 (CH), 31.5 (CH_3), 30.7 (CH_2), 30.2 (CH_2), 29.1 (CH_2), 27.9 (CH_2), 27.9 (CH_3), 26.1 (CH_2), 23.3 (CH_3), 15.0 ppm (CH_3); IR (ATR): $\tilde{\nu}$ = 3475 (w), 2956 (s), 2928 (s), 2877 (s), 1447 (m), 1374 (s), 1320 (w), 1209 (w), 1154 (s), 1126 (s), 1098 (s), 1049 (m), 937 (s), 843 (w), 804 (s), 761 (w), 541 cm^{-1} (m); MS (EI): *m/z* (%) = 222 (< 1) $[M]^+$, 204 (26), 189 (8), 161 (24), 149 (10), 134 (18), 119 (100), 105 (34), 93 (41), 79 (28), 67 (15), 59 (31), 43 (18).

Koraiol (18). R_f = 0.08 (hexane/ethyl acetate 10:1); I = 1616; $[\alpha]_D^{23}$ = +32.9 (c = 0.08, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ = 2.59 (ddd, ^3J(H,H) = 9.5 Hz, ^3J(H,H) = 9.5 Hz, ^3J(H,H) = 9.5 Hz, 1H; CH), 2.04 (ddddd, ^3J(H,H) = 12.2 Hz, ^3J(H,H) = 9.7 Hz, ^3J(H,H) = 3.7 Hz, ^4J(H,H) = 2.6 Hz, ^4J(H,H) = 1.1 Hz, 1H; CH), 1.99 (dd, ^3J(H,H) = 11.8 Hz, ^3J(H,H) = 7.8 Hz, 1H; CH), 1.95 (ddd, ^2J(H,H) = 13.2 Hz, ^3J(H,H) = 6.6 Hz, ^3J(H,H) = 1.6 Hz, 1H; CH_2), 1.79 (dddd, ^3J(H,H) = 11.8 Hz, ^2J(H,H) = 10.0 Hz, ^3J(H,H) = 10.0 Hz, ^3J(H,H) = 8.1 Hz, 1H; CH_2), 1.72 (dddd, ^2J(H,H) = 10.0 Hz, ^3J(H,H) = 7.6 Hz, ^3J(H,H) = 7.6 Hz, ^3J(H,H) = 1.4 Hz, 1H; CH_2), 1.69 (dd, ^2J(H,H) = 11.6 Hz, ^3J(H,H) = 9.3 Hz, 1H; CH_2), 1.63 (br s, 1H; OH), 1.54 (ddd, ^2J(H,H) = 10.1 Hz, ^3J(H,H) = 10.1 Hz, ^4J(H,H) = 1.0 Hz, 1H; CH_2), 1.53 (ddd, ^2J(H,H) = 11.5 Hz, ^3J(H,H) = 9.0 Hz, ^4J(H,H) = 2.5 Hz, 1H; CH_2), 1.47 (ddd, ^2J(H,H) = 9.9 Hz, ^3J(H,H) = 8.0 Hz, ^3J(H,H) = 1.4 Hz, 1H; CH_2), 1.44–1.40 (m, 2H; CH_2), 1.32 (dddd, ^2J(H,H) = 13.1 Hz, ^3J(H,H) = 12.0 Hz, ^3J(H,H) = 2.4 Hz, ^4J(H,H) = 1.1 Hz, 1H; CH_2), 1.25 (d, ^4J(H,H) = 0.8 Hz, 3H; CH_3), 1.21 (d, ^4J(H,H) = 0.8 Hz, 3H; CH_3), 1.13 (s, 3H; CH_3), 0.86 ppm (s, 3H; CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 74.0 (C_q), 53.2 (CH), 50.4 (CH), 46.3 (CH_2), 42.8 (CH), 42.3 (C_q), 37.6 (CH_2), 34.0 (CH_2), 32.3 (C_q), 31.0 (CH_3), 24.2 (CH_3), 23.4 (CH_3), 22.3 (CH_3), 22.2 (CH_2), 18.7 ppm (CH_2); IR (ATR): $\tilde{\nu}$ = 3405 (w), 2919 (s), 2859 (s), 1444 (m), 1423 (m), 1368 (s), 1310 (w), 1258 (m), 1226 (m), 1190 (m), 1110 (m), 1063 (m), 1018 (m), 993 (m), 958 (w), 935 (w), 908 (s), 888 (m), 831 (w), 814 (w), 761 (w), 630 (m), 573 (m), 540 cm^{-1} (m); MS (EI): *m/z* (%) = 222 (< 1, M^+), 207 (5), 189 (7), 179 (12), 161 (23), 149 (15), 133 (19), 121 (46), 108 (87), 93 (100), 81 (52), 71 (55), 55 (41), 43 (76).

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Keywords: biosynthesis · NMR spectroscopy · terpene cyclases · terpenoids · volatiles

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