JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Isolation and Structure Elucidation of Pentahydroxyscirpene, a Trichothecene *Fusarium* Mycotoxin

Philipp Fruhmann,[†,#] Hannes Mikula,[†,#] Gerlinde Wiesenberger,[‡] Elisabeth Varga,[§] Daniel Lumpi,[†] Berthold Stöger,[⊥] Georg Häubl,[∥] Marc Lemmens,[▽] Franz Berthiller,[§] Rudolf Krska,[§] Gerhard Adam,[‡] Christian Hametner,*[,†] and Johannes Fröhlich[†]

[†]Institute of Applied Synthetic Chemistry, Vienna University of Technology (VUT), Getreidemarkt 9/163, A-1060 Vienna, Austria

[‡]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenz Str. 24, 3430 Tulln, Austria

[§]Christian Doppler Laboratory for Mycotoxin Metabolism and Center for Analytical Chemistry, Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenz Str. 20, 3430 Tulln, Austria

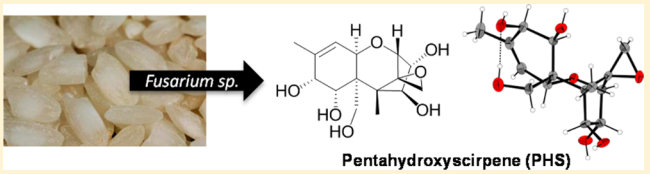
[⊥]Institute of Chemical Technologies and Analytics, Vienna University of Technology (VUT), Getreidemarkt 9/164, 1060 Vienna, Austria

[∥]Romer Laboratories Division Holding GmbH, Technopark 1, 3430 Tulln, Austria

[▽]Institute for Biotechnology in Plant Production, Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenz Str. 20, 3430 Tulln, Austria

Supporting Information

ABSTRACT: Pentahydroxyscirpene, a novel trichothecene-type compound, was isolated from *Fusarium*-inoculated rice. The structure of pentahydroxyscirpene was elucidated by 1D and 2D NMR spectroscopy and X-ray single-crystal diffraction. The conformation in solution was determined by NOESY experiments supported by quantum chemical calculations. *In vitro* toxicity tests showed that pentahydroxyscirpene inhibits protein synthesis as do other trichothecenes.



Trichothecenes, a major class of mycotoxins, are among the most prominent fungal secondary metabolites harmful to human and animal health, causing a range of acute and chronic symptoms.[1] Toxicity studies showed that trichothecenes inhibit eukaryotic protein synthesis.[2] When consumed through contaminated foods, trichothecenes are neurotoxic, immunosuppressive, and nephrotoxic.[3] Trichothecene-producing genera include *Fusarium*, *Myrothecium*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium*.[4] Within the genus *Fusarium*, some species are important plant pathogens, causing head blight in small grain cereals and ear rot in maize.[5] Surveillance studies showed that worldwide contamination of cereal grains and other feeds with *Fusarium* mycotoxins is of global concern.[6]

Over 200 trichothecenes have been reported so far, all containing a tricyclic 12,13-epoxytrichothec-9-ene core structure.[7] They are divided into four groups (types A–D) according to their chemical properties.[8] Type A trichothecenes include compounds containing a hydroxyl group (e.g., neosolaniol), an ester functionality (e.g., T-2 toxin), or no oxygen substituent at C8 (e.g., 4,15-diacetoxyscirpenol). Type B trichothecenes, such as nivalenol (**1**) or deoxynivalenol (**2**), possess a carbonyl functionality at C8 (Figure 1). Type C trichothecenes have a C7/C8 epoxide, and compounds of type D have an additional macrocycle between C4 and C15.

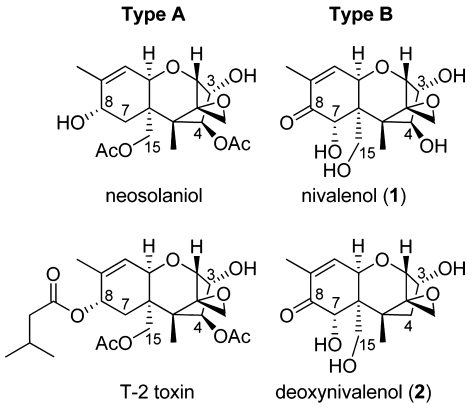


Figure 1. Structures of important type A and B trichothecenes.

Here we report the isolation and structural elucidation of pentahydroxyscirpene (**3**) (Figure 2) and results of an *in vitro* toxicity test. The conformation in solution is described as obtained by NOESY experiments supported by quantum chemical calculations. Additionally, a fast and simple procedure

Received: October 28, 2013
Published: December 24, 2013

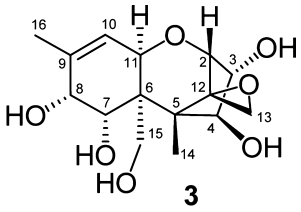


Figure 2. Structure of pentahydroxyscirpene (**3**).

for the preparation of **3** starting from nivalenol is presented, and the proposed biosynthetic pathway of the novel mycotoxin is discussed.

During isolation of nivalenol from rice inoculated with the nivalenol producing strain IFA189 following a procedure described by Krska et al.,[9] we discovered a novel trichothecene. The compound was purified as described in the Supporting Information. **3** was obtained as a crystalline, optically active compound $\{[\alpha]^{25}_{D} = -36.1$ (c 0.2; H_2O)$\}$. The molecular formula, determined by HRMS measurement of the deprotonated ion (m/z 313.1290), was calculated to be $C_{15}H_{22}O_7$ ($[M - H]^-$ calcd 313.1293), indicating 5 degrees of unsaturation. Due to four rings and one C=C double bond present in type A trichothecenes, we first considered **3** to be of classic type A structure having no carbonyl group at C-8. The ^{1}H NMR spectrum (see Supporting Information) of **3** clearly displayed two methyl groups (δ_H 1.08, 1.81), of which one is attached to an sp^2 carbon (allylic position), leading to a higher chemical shift of this signal. An olefinic proton at δ_H 5.55 indicated the presence of a trisubstituted double bond. Furthermore, 10 clearly separated signals between δ_H 2.91 and 4.42 were ascribable to protons geminal to an oxygen atom (OH or OR). The ^{13}C NMR spectrum of **3** contained 15 carbon resonances, which were further classified by APT spectra as three quarternary carbons (including one olefinic carbon at δ_C 140.1), one oxygenated quarternary carbon (δ_C 65.7), one olefinic (δ_C 123.9) and six oxygenated methines, two oxygenated methylenes (δ_C 62.0, 46.8), and two methyl groups (δ_C 20.8, 9.2). These data were consistent with the signals observed in the ^{1}H NMR spectrum. All proton signals could be unambiguously assigned to their respective carbons through analysis of the HSQC spectrum (see Supporting Information). The structure of **3** was established based on the detailed analysis of ^{1}H–^{1}H COSY, HMBC, and NOESY measurements (Figure 3).

The configuration of **3** in solution was determined, supported by quantum chemical calculations [DFT, B3LYP, 6-31++G(d,p), methanol (IEFPCM), Gaussian 09; for further details see Supporting Information] (Figure 4A). X-ray single-

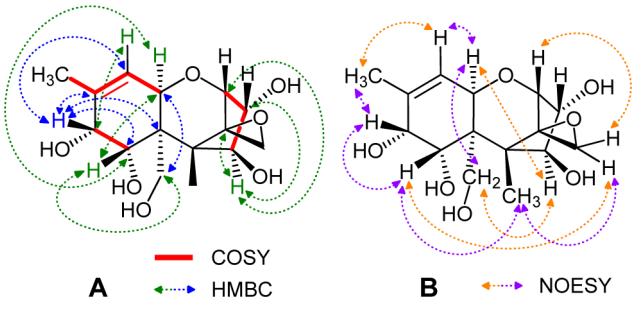


Figure 3. (A) Selected ^{1}H–^{1}H COSY, HMBC, and (B) NOESY correlations.

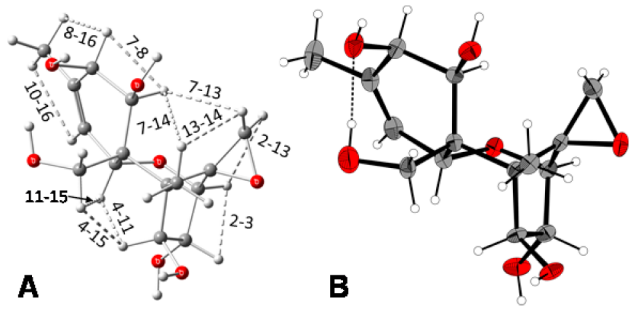


Figure 4. (A) Optimized geometry of **3** in MeOH (B3LYP, 6-311++G(d,p)) on the basis of NOESY correlations. (B) X-ray crystal structure of **3** (monohydrate; relative configuration).

crystal diffraction of **3** with Mo Kα radiation confirmed the proposed structure (Figure 4B; for further details see the Supporting Information). Furthermore UV and CD measurements were performed for comparison of **1**, **2**, and **3** (Figure 5).

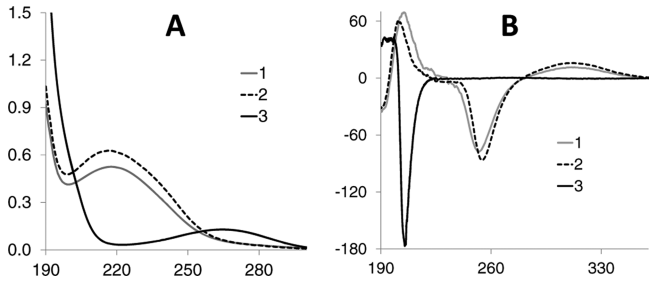


Figure 5. (A) UV and (B) CD spectra of nivalenol (**1**), deoxynivalenol (**2**), and pentahydroxyscirpene (**3**).

To elucidate the origin of **3** as well as to ensure that this compound is not an artifact or degradation product, the whole fermentation process using the same *Fusarium* strain was repeated on rice. Both nivalenol (**1**) and pentahydroxyscirpene (**3**) were detected in the cultures.

3 does not contain a chromophoric moiety (Figure 5A) and may therefore be missed by routine analysis using LC-UV.

Since **3** contains an epoxide functionality, which plays an important part in the toxicity of trichothecences,[10] this natural compound was predicted to be toxic. *In vitro* toxicity tests, chosen based on the inhibition of protein synthesis by trichothecenes, were performed with **3** as well as with **1** and **2**. Translation in rabbit reticulocyte lysates[11] was suppressed to 50% (IC_{50}) at 5.0 μM **3**, 2.0 μM **1**, and 1.8 μM **2** (Figure 6).

Furthermore, a fast and simple procedure for the synthesis of **3** starting from commercially available **1** was developed (Scheme 1A). The prepared **3** was identical (NMR, $[\alpha]^{25}_{D}$, HRMS/MS) with the isolated material, proving the absolute configuration as shown in Scheme 2A. The stereoselectivity of this Luche reduction[12] can be explained by the Cram chelation model[13] and/or substrate-controlled hydride addition (Scheme 1B).

A biogenetic pathway of **3** is proposed based on previously described routes to type A and B trichothecences (Scheme 2).[14] The biosynthetic precursor of **1** and **3** is most likely 7,8-dihydroxy-3,4,15-triacetoxyscirpenol (7,8-dihydroxy-TAS). An unknown oxidoreductase plays an important role in the production of **1** and **3**. This enzyme is predicted to catalyze the oxidation of the C8-OH, to form the acyloin moiety that is typically present in type B trichothecenes produced by various

dx.doi.org/10.1021/np4008365 | J. Nat. Prod. 2014, 77, 188–192

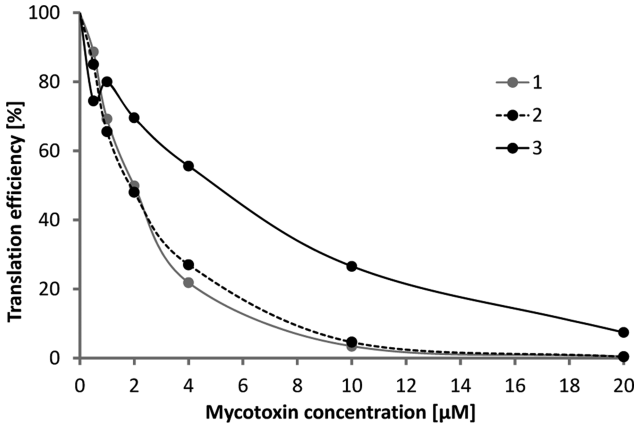
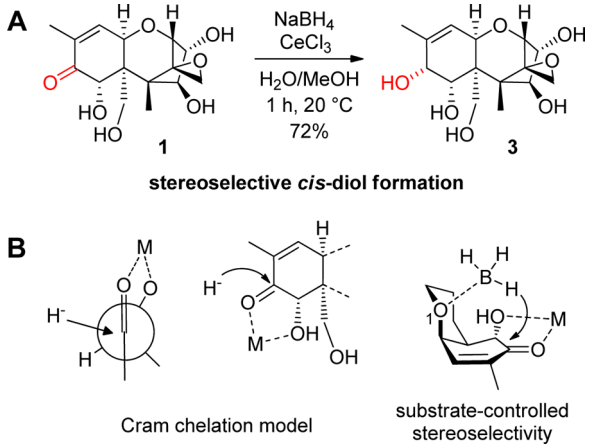


Figure 6. Inhibition of luciferase translation by **1**, **2**, and **3** (rabbit reticulocyte lysate).

Scheme 1. Stereoselective Luche Reduction of 1 to 3



Fusarium species. In case the dehydrogenation of 7,8-dihydroxy-TAS at the C8-OH is limiting, deacetylation leads to the formation of **3**.

With pentahydroxyscirpene (**3**) available, it should become possible to purify the enzyme performing the conversion of the C8-OH into a keto group leading to the biosynthesis of mycotoxins of the important class type B trichothecenes.

EXPERIMENTAL SECTION

General Experimental Procedures. Thin-layer chromatography (TLC) was performed over silica gel 60 F254 (Merck). The chromatograms were visualized by heat staining with ceric ammonium molybdate in ethanol–sulfuric acid. LC-ESIMS/MS was performed on an HCT ion trap mass spectrometer (Bruker, Germany) in full scan mode. Chromatographic separation was done on a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C_{18} column (3.0 × 150 mm, 3 μm particle size, Phenomenex, Germany), and application of pure substances was achieved using a TLC-MS interface (Camag, Germany). Preparative column chromatography was performed on silica gel 60 (40–63 μm, Merck, Germany) using a SepacoreTM Flash System (Büchi, Switzerland). HRMS and HRMS/MS spectra were obtained on a 6550 iFunnel QTOF (Agilent Technologies) using negative electrospray ionization (ESI). The 1290 Infinity UHPLC system equipped with a Zorbax SB C_{18} rapid-resolution high-definition column (150 × 2.1 mm; 1.8 μm particle size) (Agilent Technologies) was used with a methanol–water gradient containing 0.1% formic acid. ^{1}H and ^{13}C NMR spectra were recorded on an Avance DRX-400 MHz spectrometer (Bruker, Germany). Data were recorded and evaluated using TOPSPIN 1.3 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. UV spectra were recorded by using a Shimadzu UV-1800 UV spectrophotometer. Each sample was first measured within the range 190–800 nm. Since no absorption was observed in the range 400–800 nm, all samples were measured twice in the range 190–400 nm. CD spectra were recorded with a JASCO J-815 CD spectrophotometer with a N_2 monitor. The nitrogen flow was maintained at 3 L/min. After background measurement triplicates of each sample were recorded and mean values were used for visualization. Nivalenol (**1**) and deoxynivalenol (**2**) were obtained from Romer Laboratories (Tulln), and all other chemicals were purchased from Sigma-Aldrich (Austria/Germany).

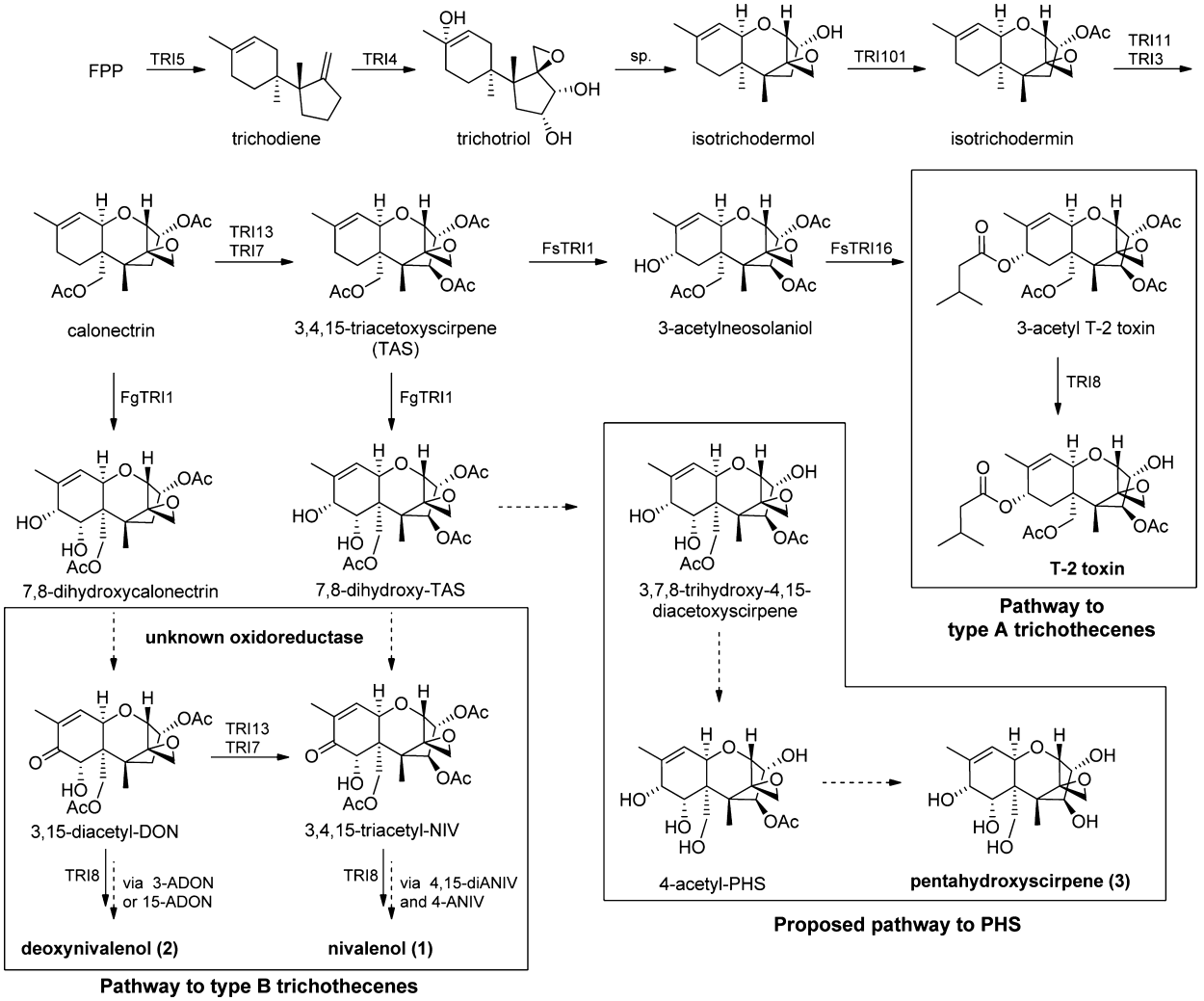
Fermentation and Purification. Following a previously described procedure,[9] the *Fusarium* strain IFA189 was cultivated in a liquid synthetic nutrient-poor medium, based on SNA (synthetic nutrient-poor agar), but without the addition of agar, for 48 h after inoculation with mycelium. Rice was inoculated with the spores and mycelium obtained suspension and incubated in the dark at 28 °C for 28 days. At the end of the incubation period, 1 kg of culture material was freeze-dried over a period of 72 h and the residue obtained was extracted with 2 L of methanol. After soaking overnight, the solution was filtered and the extraction solvent was evaporated. The remaining oily residue was dissolved in a minimum amount of methylene chloride for preparative silica gel chromatography (150 g of silica gel, $CHCl_3$–MeOH, 90:10). After application of the diluted raw extract, the substances were eluted with the same solvent mixture as described above. The eluate was collected automatically with a fraction collector, and all fractions (20 mL) were analyzed by TLC. Appropriate fractions were pooled and concentrated on a rotary evaporator to obtain a crude mixture of **1** and **3**. A 120 mg amount of this fraction of **1** and **3** was dissolved in approximately 10 mL of CH_2Cl_2–MeOH (2:1). After addition of 0.75 g of silica gel and a short shaking period the solvent was removed and the remaining material was transferred to an 8 g chromatography column, which was subsequently filled up with silica gel and connected to another 8 g silica gel column. The toxins were eluted (CH_2Cl_2–MeOH, 90:10, 10 mL/min, 5 mL fraction size), and appropriate fractions were identified by heat staining after TLC analysis (CH_2Cl_2–MeOH = 90/10, R_f(**3**) = 0.17, R_f(**1**) = 0.25).

Physical Properties of 3. Pentahydroxyscirpene (**3**): colorless crystals; mp 124–125 °C; $[\alpha]^{25}_D$ = −36.097 (*c* 0.2; H_2O); TLC (CH_2Cl_2–MeOH = 90/10) R_f 0.17; HRMS *m*/*z* 313.1290 ([M − H]$^-$, calcd for $C_{15}H_{21}O_7$, 313.1293); ^{1}H NMR (400 MHz, CD_3OD) δ 5.55 (dq, *J* = 5.6, 1.2 Hz, 1H, H-10), 4.42 (d, *J* = 5.0 Hz, 1H, H-7), 4.26 (d, *J* = 3.2 Hz, 1H, H-4), 4.14 (d, *J* = 5.9 Hz, 1H, H-11), 4.04 (t, *J* = 4.1 Hz, 1H, H-3), 3.93 (d, *J* = 12.6 Hz, 1H, H_a-15), 3.87 (d, *J* = 5.0 Hz, 1H, H-8,), 3.65 (d, *J* = 12.6 Hz, 1H, H_b-15), 3.40 (d, *J* = 4.7 Hz, 1H, H-2), 3.02 (d, *J* = 4.4 Hz, 1 H, H_a-13), 2.91 (d, *J* = 4.4 Hz, 1H, H_b-13), 1.81 (d, *J* = 1.2 Hz, 3H, CH_3-16), 1.08 (s, 3H, CH_3-14); ^{13}C NMR (100 MHz, CD_3OD) δ 140.1 (s, 1C, C-9), 123.9 (d, 1C, C-10), 82.3 (d, 1C, C-4), 81.2 (d, 1C, C-2), 80.7 (d, 1C, C-3), 72.2 (d, 1C, C-11), 72.1 (d, 1C, C-8), 71.2 (d, 1C, C-7), 65.7 (s, 1C, C-12), 62.0 (t, 1C, C-15), 50.7 (s, 1C, C-5), 49.4 (s, 1C, C-6), 46.8 (t, 1C, C-13), 20.8 (q, 1C, C-16), 9.2 (q, 1C, C-14).

Inhibition of Protein Synthesis in Rabbit Reticulocyte Lysates. The effect of **1**, **2**, and **3** on mammalian ribosomes was tested in a rabbit reticulocyte lysate-coupled *in vitro* transcription/translation system (TnT T3 coupled reticulocyte lysate system, Promega) using the newly translated luciferase as a reporter for translational activity. The transcription/translation reactions were performed in a total volume of 15 μL for 20 min according to the manufacturer's instructions in the presence of either **1**, **2**, or **3**, respectively or water as a control. The translation reactions were stopped by the addition of 1 μL of 1 mM cycloheximide. Efficiency of translation was determined by measuring activity of the firefly luciferase reporter (Steady-Glo luciferase assay system, Promega) using the 2300 EnSpireMultimode plate reader (PerkinElmer). Two independent experiments were performed using the separately produced dilutions of the mycotoxins in water.

dx.doi.org/10.1021/np4008365 | *J. Nat. Prod.* 2014, 77, 188–192

Scheme 2. Proposed Biosynthetic Pathway for 3 in Context to Previously Described Routes for Type A and B Trichothecenes[14] [a]



[a]Including already identified genes encoding an enzymatic step; FPP = farnesyl pyrophosphate, sp. = spontaneous.

X-ray Single-Crystal Diffraction (CCDC 933314). A crystal of the monohydrate of **3** was embedded in perfluorinated oil and attached to a Kapton mount. Intensity data were collected on a Bruker KAPPA APEX II diffractometer equipped with a CCD detector in ω- and φ-scan modes with 1° frame width using Mo Kα radiation. The full reciprocal sphere up to $2\theta = 60°$ was collected. The frames were reduced to intensity values using SAINT-Plus, and absorption correction was applied using the multiscan approach implemented in SADABS.[15] The structure was solved using charge flipping implemented in SUPERFLIP[16] and refined against F with JANA2006.[17] The H atoms were located in the difference Fourier maps. C−H atoms were refined as riding on the parent C atoms, and the O−H distances were restrained to 0.820(1) Å. The absolute configuration was deduced by synthesis from a precursor with known chiral centers. The monohydrate of the title compound crystallizes in space group $P2_12_12_1$ with one crystallographically unique molecule of **3** and water, respectively. As expected, the molecular conformation and the packing are defined by strong O−H···O hydrogen bonds (Table S1, Supporting Information): The molecular configuration is stabilized by one intramolecular hydrogen bond. Each molecule connects to three other molecules. The water molecule donates two and the acceptor one hydrogen bond. Thus an intricate three-dimensional network is formed.

Preparation of 3 by Stereoselective Luche Reduction of 1. Nivalenol (10.0 mg, 0.032 mmol) and $CeCl_3{\cdot}7H_2O$ (23.9 mg, 0.064 mmol) were dissolved in dry MeOH (3 mL). $NaBH_4$ (2.4 mg, 0.064 mmol) was added, and the reaction mixture was stirred for 60 min at room temperature. The solvent was evaporated, and the remaining residue was dissolved in 2 mL of H_2O−MeOH (95:5). Purification using RP-HPLC (H_2O−MeOH gradient elution) afforded 7.2 mg (72%) of **3** as a white solid. The obtained compound was identical (NMR, $[\alpha]^{25}_D$, HRMS/MS) with the isolated material.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HRMS, HRMS/MS, NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC, NOESY), X-ray crystallographic data (CIF), and details of quantum chemical calculations. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: christian.hametner@tuwien.ac.at. Tel: 0043-1-58801163607. Fax: 0043-1-5880116399.

Author Contributions

#P. Fruhmann and H. Mikula contributed equally.

Notes

The authors declare no competing financial interest.

dx.doi.org/10.1021/np4008365 | J. Nat. Prod. 2014, 77, 188−192

■ ACKNOWLEDGMENTS

The Austrian Federal Ministry of Economy, Family and Youth, the National Foundation for Research, Technology and Development, the FWF special research project SFB Fusarium, and the graduate school program Applied Bioscience Technology of the VUT in cooperation with the BOKU Vienna are gratefully acknowledged for financial support. The X-ray center of the Vienna University of Technology is acknowledged for providing access to the single-crystal diffractometer.

■ REFERENCES

(1) (a) D'Mello, J. P. F.; MacDonald, A. M. C. *Anim. Feed Sci. Technol.* **1997**, *69*, 155−166. (b) Bennet, J. W.; Klich, M. *Clin. Microbiol. Rev.* **2003**, *16*, 497−516.
(2) Cundliffe, E.; Cannon, M.; Davies, J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 30−34.
(3) (a) Rotter, B. A.; Prelusky, D. B.; Pestka, J. J. *J. Toxicol. Environ. Health* **1996**, *48*, 1−34. (b) Richard, J. L. *Int. J. Food Microbiol.* **2007**, *119*, 3−10.
(4) Ueno, Y. *Crit. Rev. Toxicol.* **1985**, *14*, 99−133.
(5) Desjardins, A. E. *Fusarium Mycotoxins, Chemistry, Genetics and Biology*; APS Press: Eagan, MN, USA, 2006; pp 1−260.
(6) Placinta, C. M.; D'Mello, J. P. F.; MacDonald, A. M. C. *Anim. Feed Sci. Technol.* **1999**, *78*, 21−37.
(7) Grove, J. F. *Prog. Chem. Org. Nat. Prod.* **2007**, *88*, 63−130.
(8) Ueno, Y.; Ishii, K.; Sato, N.; Shimada, N.; Tsunoda, H.; Sawano, M.; Enomoto, M. *Jpn. J. Pharmacol.* **1973**, *23*, 133.
(9) Krska, R.; Schothorst, R. C.; Egmond, H. P.; Josephs, R. D.; Lepschy, J.; Pettersson, H.; Chan, D.; Berthiller, F.; Schuhmacher, R.; Kandler, W.; Parich, A.; Welzig, E. *Anal. Bioanal. Chem.* **2005**, *382*, 1848−1858.
(10) Ehrlich, K. C.; Daigle, K. W. *Biochim. Biophys. Acta* **1978**, *923*, 206−213.
(11) (a) Jackson, R. J.; Hunt, T. *Methods Enzymol.* **1983**, *96*, 50−74. (b) Jagus, R.; Beckler, G. S. *Curr. Protoc. Cell Biol.* **2003**, 11.1.1−11.1.13.
(12) Luche, J.-L.; Rodriguez-Hahn, L.; Crabbé, P. *J. Chem. Soc., Chem. Commun.* **1978**, *14*, 601−602.
(13) Cram, D. J.; Elhafez, F. A. A. *J. Am. Chem. Soc.* **1952**, *74*, 5828−5835.
(14) McCormick, S. P.; Stanley, A. M.; Stover, N. A.; Alexander, N. J. *Toxins* **2011**, *3*, 802−814.
(15) *APEXII, SAINT, and SADABS*; Bruker Analytical X-ray Instruments, Inc.: Madison, WI, USA, 2008.
(16) Palatinus, L.; Chapuis, G. *J. Appl. Crystallogr.* **2007**, *40*, 786−790.
(17) Petříček, V.; Dušek, M.; Palatinus, L. *Jana 2006*, The Crystallographic Computing System; Institute of Physics: Praha, Czech Republic, 2006.