Trichodion, a New Bioactive Pyrone from a *Trichosporiella* species

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Trichodion (**1a**), a new bioactive pyrone derivative was isolated from submerged cultures of a *Trichosporiella* species in the course of a screening for inhibitors of the IFN-γ mediated signal transduction in HeLa S3 cells. The compound inhibited the IFN-γ mediated expression of the reporter gene secreted alkaline phosphatase (SEAP) with IC_{50} values of 21~42 μM (5~10 μg/ml). In addition, the expression of a human TNF-α promoter driven luciferase reporter gene was inhibited with IC_{50}-values of 10~21 μM (2.5~5 μg/ml). The structure of trichodion (**1a**) was elucidated by spectroscopic methods.

The expression of many proinflammatory enzymes (*e.g.* COX-2, NOS II), acute phase proteins and cytokines (*e.g.* TNF-α) is regulated by different transcription factors like NF-κB, AP-1 or STAT (signal transducers and activators of transcription) molecules which are activated by a variety of inducing agents like bacterial lipopolysaccharide (LPS), tumor promotors or cytokines (*e.g.* IFN-γ, IL-6). Inhibitors which specifically interfere with components of different intracellular signaling pathways or inhibit the activation of transcription factors responsible for the expression of disease-related genes may have applications as novel therapeutics in inflammation[1]. In order to search for new inhibitors of inflammatory signal transduction pathways, inducible reporter gene vectors were constructed and submerged cultures of basidiomycetes, ascomycetes and fungi imperfecti were examined for the production of compounds which inhibit the stimulus dependent JAK/STAT or human TNF-α promoter mediated expression of the reporter genes in HeLa S3 or Jurkat cells. A screening of some 500 strains of basidiomycetes, ascomycetes and fungi imperfecti resulted in the isolation of trichodion from fermentations of the *Trichosporiella* species 20-95. In the following paper we report the fermentation of the producing strain, the isolation, structural elucidation and some biological activities of the compound.

Experimental

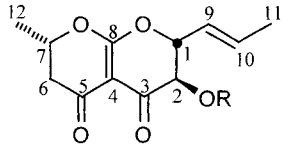
General Experimental Section

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$=145 Hz and $^nJ_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting point (uncorrected) were determined with a Reichert microscope, and the optical rotation measured with a Perkin-Elmer 141 polarimeter at 22°C.

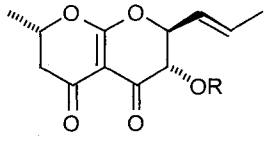
Trichodion (**1a**) was obtained as colourless crystals, m.p. 167~169. $[\alpha]_D$ +75° (*c* 0.8 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 263 nm (8,700). IR (KBr): 3460, 2980, 1730, 1660, 1565, 1445, 1300, 1235, 1160, 1045, 985 and 940 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): 6.07, dq, $J_{9\text{-}10}$=15.5, $J_{10\text{-}11}$=6.4, 10-H; 5.71, dd, $J_{1\text{-}9}$=7.5, $J_{9\text{-}10}$=15.5, 9-H; 4.66, dd, $J_{1\text{-}2}$=12.3, $J_{1\text{-}9}$=7.5, 1-H; 4.55, m, 7-H; 4.12, d, $J_{1\text{-}2}$=12.3, 2-H; 2.71, dd, $J_{6a\text{-}6b}$=17.3, $J_{6a\text{-}7}$=11.1, 6-Ha; 2.57, dd, $J_{6a\text{-}6b}$=17.3, $J_{6b\text{-}7}$=3.5, 6-Hb; 1.84, d, $J_{10\text{-}11}$=6.4, 11-H$_3$; 1.46, d, $J_{7\text{-}12}$=6.3, 12-H$_3$. ^{13}C NMR (125 MHz, $CDCl_3$): 188.5 C-3; 181.7 C-5; 160.6 C-8; 135.5 C-10; 124.6 C-9; 102.3 C-4; 84.7 C-1; 70.8 C-7; 70.3 C-2; 35.4 C-6; 20.6 C-12; 19.0 C-11. EIMS (70 eV), *m/z* (rel. int.): 238.0855 (10%, M^+, $C_{12}H_{14}O_5$ requires 238.0841), 223 (5%), 155 (17%), 84 (100%), 69 (28%), 55 (23%).

The acetylation of trichodion (**1a**) with acetic anhydride in pyridine (5 mg + 0.1 ml in 1 ml) yielded a 4 : 3 mixture of acetyltrichodion (**1b**) and the isomer **1c** as major product (65% yield). The mixture was obtained as a colourless oil with $[\alpha]_D$ +207° (*c* 0.3 in $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): 6.00, m, 10-H; 5.56, m, 9-H; 5.34/5.29, d, $J_{1\text{-}2}$=11.6/9.9, 2-H; 4.99/4.92, dd, $J_{1\text{-}2}$=11.6/9.9,

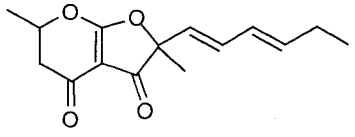
Fig. 1. Structures of trichodion (**1a**), acetyltrichodion (**1b**, **1c**) and cyclogregatin (**2**).



1a R = H
1b R = Ac



1c



2

$J_{1\text{-}9}$=8.0/7.5, 1-H; 4.54, m, 7-H; 2.69/2.68, dd, $J_{6a\text{-}6b}$=17.5/18.0, $J_{6a\text{-}7}$=11.0/11.7, 6-Ha; 2.59/2.58, dd, $J_{6a\text{-}6b}$=17.5/18.0, $J_{6b\text{-}7}$=3.7/3.5, 6-Hb; 2.15/2.14, s, 2-OAc, 1.78/1.79, dd, $J_{9\text{-}11}$=1.5/1.5, $J_{10\text{-}11}$=6.4/6.4, 11-H_3; 1.47/1.46, d, $J_{7\text{-}12}$=6.2/6.2, 12-H_3. ^{13}C NMR (125 MHz, $CDCl_3$): 181.6/181.7 C-3; 180.3/180.8 C-5; 160.3/160.3 C-8; 136.3/136.1 C-10; 123.5/123.1 C-9; 103.6/103.6 C-4; 82.8/82.8 C-1; 70.8/70.7/70.6/70.6 C-2 and C-7; 35.5/35.3 C-6; 20.6/20.5/20.4/20.3 C-12 and Ac-CH_3; 18.0/17.9 C-11. EIMS (70 eV), *m/z* (rel. int.): 280.0944 (8%, M^+, $C_{14}H_{16}O_6$ requires 238.0947).

Producing Strain

The deuteromycete strain 20-95 was isolated from a soil sample collected in Germany. The specimen showed the characteristics of the genus *Trichosporiella* as described by DOMSCH *et al.*[2]. The species however could not be identified. The strain was kindly provided by H. ANKE and is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Cultivation and Isolation

For maintenance on agar slants the strain was kept on YMG medium[3]. For submerged cultivation, strain 20-95 was grown in malt extract medium (40 g/liter malt extract). A well-grown seed culture of *Trichosporiella* sp. 20-95 (200 ml YMG medium) was used to inoculate a Biolafitte C-6 fermenter containing 20 liters of malt extract medium with aeration (3 liters air/minute) and agitation (120 rpm) at 22°C. The production of trichodion was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluid in the reporter gene assays as described below. After nine days the culture fluid was separated by filtration and applied onto Mitsubishi DIAION HP 21 resin. The resin was washed with water and the active compound was eluted with acetone. The eluate was concentrated and the remaining aqueous residue extracted with EtOAc. The solvent was evaporated and the crude product (1.16 g) was separated by chromatography on silica gel (Merck 60) with cyclohexane : EtOAc (50 : 50) as eluent resulting in 155 mg of an enriched product. Precipitation from acetonitrile yielded 62 mg of pure trichodion.

Biological Assays

HeLa S3 (ATCC CCL 2.2.) cells were maintained in DMEM-medium supplemented with 10% fetal calf serum (FCS) and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. Jurkat (ATCC TIB 152) cells were grown in RPMI 1640 medium with 10% FCS. The assays for antimicrobial activity and cytotoxicity were carried out as described previously[4]. The reporter plasmid pGE3-GAS/ISRE was constructed essentially as described earlier by cloning five copies of an GAS/ISRE consensus oligonucleotide (5′-AAGTACTTTCAGTTTCATATTACTCTA-3′) immediately upstream of the thymidine kinase promoter driven SEAP reporter gene[5]. The 1.2 kb human TNF-α promoter was amplified by PCR from genomic DNA extracted from HeLa S3 cells as described recently[6]. The PCR product was cloned into the *Xho*I-*Hin*dIII site of pGL3-Basic (Promega) to generate the TNF-α promoter driven luciferase reporter plasmid (pJR-TNFpro). The plasmid pCH110 for normalizing transfection efficiency was obtained from Amersham. Transfection of HeLa S3 cells and determination of the activity of the expressed SEAP was performed as described previously. The transfection of Jurkat cells was performed by electroporating (BioRad, Gene Pulser) 6×10^7 cells/ml in 0.2 ml HEBS buffer (10 mM HEPES, pH 7.05, 68.5 mM NaCl, 2.5 mM KCl, 0.35 mM Na_2HPO_4, 3 mM dextrose) together with 50 μg of the pJR-TNFpro plasmid at 500 V/cm. After electroporation the cells were seeded at 1×10^6 cells/ml OPTI-MEM containing 10% FCS in a 24 well plate with and without test compounds and luciferase expression was induced with 32 nM TPA and 2 μM ionomycin. The activity of the luciferase in whole cell extracts was determined 24 hours after transfection using the luciferase assay system (Promega) according to the manufacturer's instructions with a luminometer.

Fig. 2. Pertinent HMBC correlations observed with trichodion (**1a**).

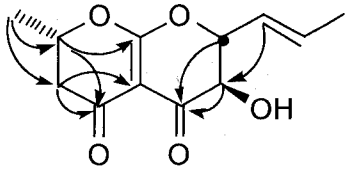
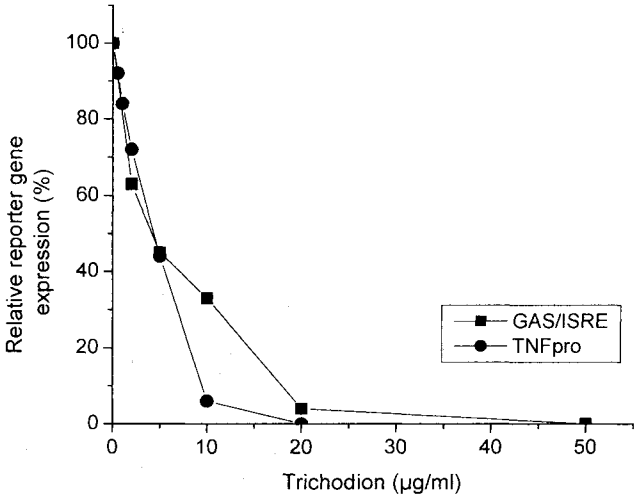


Fig. 3. Effect of trichodion on SEAP and luciferase reporter gene expression in HeLa S3 or Jurkat cells.



Hela cells were transfected with a GAS/ISRE (5XGAS/ISRE-SEAP) dependent SEAP reporter plasmid and stimulated with 10 ng/ml IFN-γ for 48 hours with or without trichodion. Jurkat cells were transfected with a hTNF-α promoter dependent luciferase reporter plasmid and stimulated with 32 nM TPA and 2 μM ionomycin with or without trichodion for 24 hours. The expression of the reporter genes were determined as described in the Experimental Section.

Results and Discussion

Trichodion (**1a**) was isolated from the crude extract of the culture fluid of *Trichosporiella*, strain 20-95 by bioactivity-guided fractionation as described in the experimental section. The EI mass spectrum suggested that the molecular weight of **1a** is 238, and high resolution measurements indicated that the elemental composition is $C_{12}H_{14}O_5$. 12 signals are visible in the ^{13}C NMR spectrum and signals integrating for 11 protons in the ^1H NMR spectrum, confirming the suggested elemental composition and establishing that one proton is exchangeable. Acetylation of **1a** with acetic anhydride in pyridine resulted in the acetylation of 2-O, as a mixture of acetyltrichodion **1b** and it's epimer **1c** was obtained. In the mass spectrum, the molecular ion of the mixture is found at *m/z* 280, and the exact mass corresponds to the elemental composition $C_{14}H_{16}O_6$. The co-formation of the epimer **1c**, which NMR data are very similar to those of **1b**, is presumably the result of a series of rapid enolisations in pyridine prior to acetylation. In both **1b** and **1c** (as in **1a**, *vide infra*), the large ^1H-^1H coupling constants between 1-H and 2-H, as well as between 6-Ha and 7-H, show that the substituents on C-1, C-2 and C-7 all are pseudoequatorial and that **1c** is not simply the C-2 epimer of **1b**. The index of hydrogen deficiency of **1a** is consequently 6, and as the NMR data suggest that **1a** contains two carbon–carbon double bonds and two keto groups it should also contain two rings. COSY and HMBC correlations establish the proton spin systems 12-H_3/7-H/6-H_2 and 11-H_3/10-H/9-H/1-H/2-H (see Figure 2 for pertinent HMBC correlations), and weak HMBC correlations between 7-H and C-8, and between 6-H_2 and C-4 determine the left ring. C-1 is obviously oxygenated, according to it's ^{13}C NMR shift, and HMBC correlations between both 1-H and 2-H to C-3 show that the second keto function is positioned next to C-2. The resulting two fragments include all atoms of **1a**, and the second ring must consequently be formed by joining O-1 with C-8 and C-3 with C-4, or O-1 with C-4 and C-3 with C-8. No HMBC correlations from either 1-H to C-8 or from 2-H to C-4 to support the suggested structure were observed, but only this alternative is in agreement with the ^{13}C NMR shifts of C-4 and C-8. Cyclogregatin (**2**), which has a similar bicyclic structure, has almost the identical ^{13}C NMR shifts of the tetrasubstituted double bond[7]. The large ^1H-^1H coupling constant between 1-H and 2-H, as well as between 6-Ha and 7-H (12.3 and 11.1 Hz, respectively), suggest that the four protons are pseudoaxial and that the two pairs of protons are *trans*. This is confirmed by the NOESY correlations between 9-H and 1-H as well as 2-H, and between 7-H and 6-Hb. Although the two spin systems is rather far from each other, a weak NOESY correlation could be observed between 2-H and 6-Ha suggesting that the two are situated on the same side of the molecule. No attempts were made to determine the absolute configuration of **1a**.

Trichodion has been detected during fermentation and

isolation using a SEAP reporter plasmid containing multiple GAS/ISRE sites in the promoter (pGE3-GAS/ISRE) and a human TNF-α promoter driven luciferase reporter plasmid (pJR-TNFpro). Transfection of Hela S3 cells with the vector pGE3-GAS/ISRE and stimulation with 10 ng/ml IFN-γ resulted in a 8~10 fold increase in SEAP expression and transfection of Jurkat cells with the vector pJR-TNFpro and stimulation with TPA/Ionomycin resulted in a 8 fold increase in luciferase expression compared to the uninduced control. Trichodion inhibited the IFN-γ induced SEAP expression with IC_{50} values of 21~42 μM (5~10 μg/ml; Fig. 3). In addition, the expression of the human TNF-α promoter driven luciferase reporter gene was inhibited with IC_{50}-values of 10~21 μM (2.5~5 μg/ml). Trichodion exhibited no antibacterial or antifungal activities. Cytotoxic activities could be observed starting from 50 μg/ml. Further biological activities and the mode of action of trichodion will be published elsewhere[8].

Acknowledgments

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References

1) MANNING, A. M.: Transcription factors: a new frontier for drug discovery. DDT 1: 151~160, 1996
2) DOMSCH, K. H.; W. GAMS & T.-H. ANDERSON: Compendium of soil fungi. Volume 1, 1993
3) ERKEL, G.; T. ANKE, R. VELTE, A. GIMENEZ & W. STEGLICH: Hyphodontal, a new antifungal inhibitor of reverse transcriptases from *Hyphodontia* spec. (Corticiaceae, Basidiomycetes). Z. Naturforsch. 49c: 561~570, 1994
4) WEBER, W.; T. ANKE, B. STEFAN & W. STEGLICH: Antibiotics from basidiomycetes. XXXII. Strobilurin E: A new cytostatic and antifungal *E*-β-methoxyacrylate antibiotic from *Crepidotus fulvotomentosus* Peck. J. Antibiotics 43: 207~212, 1990
5) ERKEL, G.; T. ANKE & O. STERNER: Inhibition of NF-κB activation by panepoxydone. Biochem. Biophys. Res. Commun. 226: 214~221, 1996
6) TAKASHIBA, S.; L. SHAPIRA, S. AMAR & T. E. VAN DYKE: Cloning and characterization of human TNF alpha promoter region. Gene 131: 307~308, 1993
7) ANKE, H.; I. CASSER, M. SCHRAGE & W. STEGLICH: Cyclogregatin, a new metabolite from *Aspergillus panamensis*. J. Antibiotics 41: 1681~1684, 1988
8) ERKEL, G.: Trichodion, a new inhibitor of inflammatory signal transduction pathways from a *Trichosporiella* species. FEBS Letters 477: 219~223, 2000