Pergamon

Tetrahedron Letters, Vol. 37, No. 36, pp. 6539-6542, 1996

0040-4039/96 $15.00 + 0.00

PII: S0040-4039(96)01445-1

Fischerellin A, a Novel Photosystem-II-inhibiting Allelochemical of the Cyanobacterium *Fischerella muscicola* with Antifungal and Herbicidal Activity

Leonhard Hagmann[1] and Friedrich Jüttner[2,*]

[1] Crop Protection Research, SANDOZ AGRO Ltd., Bldg. 94/314, CH-4002 Basel, Switzerland;
[2] Institute of Plant Biology / Limnology, University of Zürich, Limnological Station, Seestr. 187, CH-8802 Kilchberg, Switzerland.

Abstract. Fischerellin A, the most active allelochemical compound of *Fischerella muscicola* that is directed against other cyanobaceria and photoautotrophic organisms, is a potent photosystem-II inhibitor of novel structure. Structural elements are an enediyne moiety and two heterocyclic ring systems. Fischerellin A is unique and unrelated to all known cyanobacterial metabolites, it exhibits a MIC of 14 nM against *Synechococcus* PCC 6911, and has interesting antifungal and herbicidal activity.

Cyanobacteria are archaic photosynthetic microorganisms which have flourished on earth for more than three billion years with some genera showing only minor morphological changes since that time.[1] Based upon the assumption that this group of microorganisms has preserved enzymatic properties and reaction sequences to synthesise structural elements that have been lost during development of higher organised plants, an extensive screening was initiated in recent years to identify such compounds. The screening was preferentially performed with pharmacological important assays in which cytotoxicity was the basic criterion for further investigations. The screening for secondary metabolites that are inhibitory against other photoautotrophs (cyanobacteria, algae and higher plants) has only scarcely been carried out although the knowledge of such compounds would be important in the development of new degradable herbicides, and for the understanding of allelopathic reactions in ecosystems. The identification of cyanobacterin[2] and hapalindole A[3] demonstrates the existence of such cyanobacterial secondary metabolites. In a recent study we reported on the biological and biochemical features of fischerellin, which is the major anticyanobacterial and algicidal agent of *Fischerella muscicola*.[4] Here we report the characterisation and structure elucidation of this cyanobacterial secondary metabolite, fischerellin A (**1**), which is also shown to be a potent photosystem-II inhibitor.

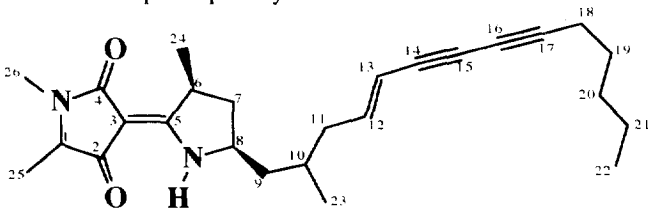

Figure 1. Structure of Fischerellin A (1).

The origin and cultivation of axenic *Fischerella muscicola* UTEX 1829 have been described.[4] The cyanobacterial biomass (94 g), which was collected from four 6 L glass tube reactors after 28 days, was separated from the medium and extracted twice with 60% methanol. *Tert.* butylmethylether and water were added to the methanolic extract, and the ether phase separated[5] and evaporated *in vacuo*. The residue was applied to a C-18 cartridge in order to remove the chlorophylls.[6] The evaporated eluate was then purified twice on a C-18 reversed phase HPLC column,[7] which yielded 5 mg pure fischerellin A (**1**) as colourless powder.

Table 1. NMR Shifts and Correlations of Fischerellin A in $CDCl_3$

Carbon Number	Carbon Shift [a,d]	Multiplicity	Proton Shift [b,d]	COSY Correlations[d]	HMBC Correlations [c,d]
1	61.8 *61.0*	CH	3.58 *3.61*	1.31 *1.34*	2.93, 1.31 *2.92, 1.34*
2	*198.8* 194.3	C	- -	- -	*3.61, 1.34* 3.58, 1.31
3	*93.3* 91.6	C	- -	- -	- -
4	172.8 *169.2*	C	- -	- -	2.93 *2.92*
5	*173.9* 173.7	C	- -	- -	*1.47* 1.41
6	38.2 *37.8*	CH	3.56 *3.67*	2.47, 1.48, 1.41 2.47, 1.48, *1.47*	9.67, 1.41 *9.94, 1.47*
7	34.6 *34.5*	CH_2	2.47 1.48	*3.85*, 3.83, *3.67*, 3.56, 1.48 *3.85*, 3.83, *3.67*, 3.56, 2.47	9.67, 3.83, 1.41 *9.94, 3.85,1.47*
8	*60.4* 60.1	CH	*3.85* 3.83	*9.94*, 1.63, 1.58 9.67, 1.63, 1.58	*9.94*, 2.47, 1.63 9.67, 2.47, 1.63
9	37.7 *37.6*	CH_2	1.63 1.58	1.44 1.44	3.83, 2.47, 1.48 *3.85*, 2.47, 1.48
10	28.3	CH	1.44	2.14, 1.63, 1.58, 1.42	2.14, 1.42
11	32.9	CH_2	2.14	6.24, 1.44	6.24, 5.49, 1.44
12	147.1	CH	6.24	5.49, 2.14	5.49, 2.14, 1.42
13	109.3	CH	5.49	6.24, 2.31	2.14
14	73.7	C	-	-	6.24, 5.49
15	73.2	C	-	-	5.49, 2.31
16	65.1	C	-	-	5.49, 2.31
17	84.0	C	-	-	2.31, 1.54
18	19.5	CH_2	2.31	5.49, 1.54	1.54
19	28.0	CH_2	1.54	2.31, 1.38	2.31
20	31.0	CH_2	1.38	1.54, 1.32	2.31, 1.54, 1.32, 0.90
21	22.1	CH_2	1.32	1.38, 0.90	1.54, 1.38, 0.90
22	13.9	CH_3	0.90	1.32	1.32
23	26.1	CH_3	1.42	1.44	2.14, 1.63, 1.44
24	*21.6* 20.7	CH_3	*1.47* 1.41	*3.67* 3.56	*3.67*, 2.47, 1.48 3.56, 2.47, 1.48
25	*15.4* 15.3	CH_3	*1.34* 1.31	*3.61* 3.58	*3.61* 3.58
26	*26.5* 26.3	CH_3	*2.92* 2.93	- -	- -
NH	-	-	*9.94* 9.67	*3.85* 3.83	- -

[a] 500 MHz, assignments by the HMQC method. [b] 125 MHz. [c] optimised for $^{n}J_{CH} = 8$ Hz.
[d] The *italic* figures correspond to the minor tautomer. The observed ratio of the tautomers at r.t. is about 3:2 .

Fischerellin A shows a strong molecular ion at m/z 408 (EI-MS), and a strong protonated molecular ion at m/z 409 (MH^+) in the thermospray mass spectrum.[8] High resolution FAB-MS revealed the molecular formula $C_{26}H_{36}N_2O_2$ (409.2844 for MH^+, difference to the calculated mass: 1.1 mmu). The nitrogen content of the molecular formula was confirmed by the analysis of ^{15}N-labeled compound[9] which showed a molecular ion shift of 2 amu to m/z 410 (EI-MS). For better signal intensities, ^{13}C-labeled fischerellin was produced for NMR spectroscopy.[10]

Fischerellin A has a very characteristic UV absorption spectrum with λ_{max} (ε) of 214 (53'000), 229 (12'000), 241 (16'000), 253 (18'000), 268 (20'600), 284 (24'000) and 301 (22'000) nm. The hydrogenation (H_2/Pt) shifted the mass to m/z 418 (EI-MS) indicating the presence of five reduceable double bond equivalents (DBE). Comparison of literature data of unsaturated polyacetylenes[11,12] gave strong evidence for the presence of an endiyne. This corresponds to the quaternary carbons at 84.0, 65.1, 73.2 and 73.7 ppm for the two triple bonds, and the trans double bond signals at 109.3 and 147.1 ppm with proton shifts of 6.24 and 5.49 ppm ($J_{12,13}$=16.2 Hz). The latter also exhibits long range H/C correlations to the acetylene part. The methylene group next to the triple bond (2.31 ppm) shows a typical high field shift (19.5 ppm) and 2D correlations (COSY, HMQC, HMBC) strongly suggest the attachment of an n-pentyl chain to the triple bond system which is also supported by typical MS fragments.[13] Five DBE are involved in this part of the molecule, the remaining five DBE of a total of ten are an unsaturated ketone (194.9/*198.8* ppm), an amide (172.8/*169.2* ppm), an unusual double bond (173.7/*173.9* ppm and 91.6/*93.3* ppm) and two ring systems. As the compound was shown to be analytically pure (GC and HPLC), the doubling of all NMR signals in this region was attributed to restricted rotation as illustrated in Figure 2 with a rotational tautomerism. Extensive COSY, HMQC and HMBC measurements allowed the assignment of all these signals and correlations (see Table 1). The enamine proton can easily form hydrogen bonds to either the ketone or the amide carbonyl which explains its down field shift (9.67, *9.94* ppm). The unusual C3-C5 double bond shifts can be explained by the effects of the two carbonyls and the amine function on this double bond. This conjugated system is responsible for the UV absorption at 301 nm which remained intact after hydrogenation. Chemical carbon shift estimations[14] of Fischerellin A are in excellent agreement with the measured values. The relative configuration of the substituents at C6 and C8 on the five-membered ring was secured from the strong ROESY correlations of the adjacent ring protons.[15]

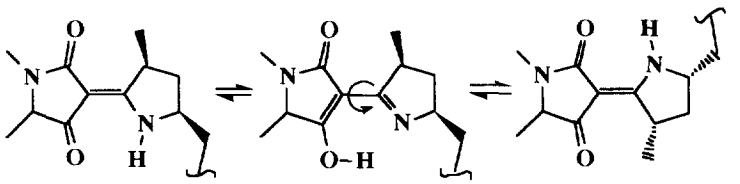

Figure 2. Rotational tautomerism of Fischerellin A.

The bioassays designed for toxicity determinations against cyanobacteria, green algae, rotifera and crustaceans have been described previously.[16] Fischerellin A was strongly inhibitory against axenic cyanobacterial cultures of *Anabaena* PCC 7120, *Anabaena* P-9 and *Synechococcus* PCC 6911. The MIC for *Synechococcus* PCC 6911 in a 54 hours growth assay was 14 nM. The green algae *Scenedesmus obliquus* SAG 276-1 and *Scenedesmus subspicatus* SAG 86.81 were less sensitive, exhibiting a MIC of 0.5 μM. The LC_{50} (24 hrs.) for the rotifer *Brachionus calyciflorus* was 4 μM, and 6 μM for the crustacean *Thamnocephalus platyurus*.

As reported earlier,[4] the strong inhibition of the photosystem-II has been confirmed with higher plants. Herbicidal effects have been demonstrated against *Lemna minor* at 50 µM (60% PS-II inhibition, 44% growth inhibition), whereas at 100 µM, the photosystem of this plant is almost totally blocked (98% PS-II inhibition, 74% growth inhibition).

Fungicidal acivities have been shown in an assay[17] against several agronomically important microorganisms which can severely affect all kinds of crop plants. Fischerellin A exhibits total growth inhibition (100%) of brown rust *(Uromyces appendiculatus* on bean) already at 250 ppm, whereas powdery mildew *(Erysiphe graminis* on barley) shows 100% growth inhibition at 1000 ppm. Downy mildew (late blight, *Phytophthora infestans* on tomato) and rice blast *(Pyricularia oryzae)* display 80% inhibition at 1000 ppm. Less activity (30% growth inhibition at 1000 ppm) was observed against brown rot (blossom blight, *Monilinia fructigena)* and stem break *(Pseudocercosporella herpotrichoides)* grown on agar.

ACKNOWLEDGMENT: The technical assistance of C. Lehmann is gratefully acknowledged. We also thank L. Oberer (NMR), C. Quiquerez (HR-MS), R. Gees (antifungal tests) and R.Chollet (herbicidal tests) for their respective measurements and A.J.F. Edmunds for correcting the manuscript. This study was supported by a grant of the Swiss National Science Foundation, Bern, to F.J.

REFERENCES AND NOTES

1. Schopf, J.W. *Proc.Natl.Acad.Sci. USA* **1994**, *91*, 6735-6742.
2. Mason, C.P.; Edwards, K.R.; Carlson, R.E.; Pignatello, J.; Gleason, F.K.; Wood, J.M. *Science (Wash.)* **1982**, *215*, 400-402.
3. Moore, R.E.; Cheuk, C.; Patterson, G.M.L. *J.Am.Chem.Soc.* **1984**, *106*, 6456-6457.
4. Gross, E.M.; Wolk, C.P.; Jüttner, F. *J.Phycol.* **1991**, *27*, 686-692.
5. The biomass was separated with a strainer. Its methanolic (60%, 280 mL) extract was partitioned between 100 mL TBME and 200 mL water.
6. The residue was dissolved in MeOH/EtOH/H_2O 45:45:10 and passed through the C-18 cartridge (Mega-BondElut, Varian), eluted with the same solvent mixture and collected before green compounds appeared.
7. HPLC: 250x10 mm, Spherisorb S5 ODS-2 (PhaseSep), linear gradient elution (3 mL/min.) from 50% methanol in water to 100% methanol in 12 min. and kept at 100% methanol for 10 min. Fischerellin A eluted after 15.6 min. under these conditions. The second chromatography was isocratic with acetonitrile/water 80:20 (same column and flow rate). The lipophilic nature of Fischerellin A also became evident by the 1-octanol/water partition constant of log K_{ow} = 3.6 at 25 °C.
8. Low resolution EI-MS were obtained on a GC-MS after separation on a 10 m capillary column (0.32 mm i.d., SIM-DIST-CB, Chrompack, helium carrier gas): m/z (rel. int. %) 408 (100), 365 (35), 351 (40), 337 (35), 242 (43), 207 (94), 176 (71), 148 (41), 94 (48). Thermospray MS were recorded on a Vestec mass spectrometer (1 ml/min., methanol / 0.1 M aqueous ammonium acetate 1:1).
9. High percentage labeling of Fischerellin A was obtained by replacing nitrate of 5 L cyanobacterial medium by 99% ^{15}N-nitrate (4 mM). *F. muscicola* grown in this medium for 28 days was producing 29 g biomass. 27% of the Fischerellin molecules were single-labeled, 66% were double-labeled (shown by MS).
10. To obtain low percentage ^{13}C-labeled Fischerellin, each of three 6 L glass tube reactors were continuously gassed with compressed air, supplemented twice a day with 5 mL of a $NaH^{13}CO_3$ solution (3 mg/mL, pH 9.5), added through a sterile filter. The harvest after 30 days yielded 91 g wet biomass, from which 1.5 mg ^{13}C-labeled Fischerellin A were obtained. The atom percentage labeling was 3.5 % (by MS).
11. Ahmed, M.; Barley, G.C.; Bentley, R.K.; Hearn, M.T.W.; Higham, C.A.; Jenkins, J.K.; Jones, Sir E.R.H.; Keeping, J.W.; Thaller, V.; Yates, J.A. *J. Chem. Soc. Perkin I* **1974**, 1981-1994.
12. Wittstock, U.; Hadacek, F.; Wurz, G.; Teuscher, E.; Greger, H. *Planta Med.* **1995**, *61*, 439-445.
13. m/z 379, 365, 351, 337 and 29, 43, 57, respectively.
14. Pretsch E. ^{13}C-Shift-Estimation. In: *SpecTool, a Hypermedia Book for Structure Elucidation of Organic Compounds with Spectroscopic Methods*, Chemical Concepts, Weinheim, **1995**.
15. H-C6 and H-C8 give strong ROESY correlations with one of the C7 methylene protons at 2.47 ppm, indicating that all these protons are on the same side of the five-membered ring, hence both ring substituents must be *cis* to each other.
16. Todorova, A.; Jüttner, F. *Phycologia* (in press).
17. Niderman, T.; Genetet, I.; Bruyère, T.; Gees, R.; Stintzi, A.; Legrand, M.; Fritig, B.; Mösinger, E. *Plant Physiology* **1995**,*108*, 17-27. Schweizer, P.; Gees, R.; Mösinger, E. *ibid.* **1993**, *102*, 503-511.

(*Received in Germany* 14 *May* 1996; *accepted* 19 *July* 1996)