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NOTE

Graminin B, a furanone from the fungus *Paraconiothyrium* sp.

Celso Almeida, Noureddine El Aouad, Jesús Martín, Ignacio Pérez-Victoria, Víctor González-Menéndez, Gonzalo Platas, Mercedes de la Cruz, Maria Cândida Monteiro, Nuria de Pedro, Gerald F Bills, Francisca Vicente, Olga Genilloud and Fernando Reyes

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Members of the furanone structural class of natural oxygenated heterocycles have been reported from fungi of the genera *Cephalosporium* (for example, gregatins and graminin A), *Aspergillus* (huaspenone A and B or the unnamed tetronic acid derivatives from *A. panamensis*) and *Penicillium* (penicilliol).[1–6] The structure initially proposed of 3-acyl-4-methoxyfuran-2(5*H*)-one was later revised to a structure of 4-acyl-5-methoxyfuran-3(2*H*)-one after the total synthesis of racemic and (+)-gregatin B by Takaiwa and Yamashita.[7,8] Very recently, a serendipitous synthesis of (+)-gregatin B[9] and total syntheses of the gregatins A–D and aspertetronin A[10] led to a second structural revision establishing a central core of 4-(methoxycarbonyl)furan-3(2*H*)-one as the right structure for the members of this family of secondary metabolites (Figure 1).

As part of a continuing program to identify novel pharmaceutical lead structures from natural sources, Fundación MEDINA has been investigating new antibiotic agents from its proprietary library of over 100 000 strains of actinomycetes and fungi. The fermentation extract of a fungal strain was selected for further purification based on its antibiotic activity. A phylogenetic placement of this strain, based on its ITS/28S, identified the fungus as a species of *Paraconiothyrium* and closely related to *Paraconiothyrium hawaiensis*. Herein we report the isolation and structural elucidation of graminin B (**1**), a new furanone derivative obtained from fermentation broths of this species, and its activity against *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*.

Graminin B was isolated from acetone extracts of *Paraconiothyrium* sp. (CF-217411) by fractionation on SP207SS resin followed by repeated semi-preparative HPLC. The molecular formula of **1** was deduced to be $C_{18}H_{26}O_4$ by accurate mass measurement (ESI-TOF, Supplementary Figure S4), requiring six degrees of unsaturation. The ^{13}C NMR and $^{1}H/^{13}C$ HSQC spectroscopic analysis indicated the presence of 18 carbon resonances, including four resulting from methyl groups and four from sp^2 methines, whereas further five signals resulting from methylene groups and five resonances were assigned to quaternary carbons (see Table 1 and Supplementary Figure S2).

The ^{1}H NMR (Supplementary Figure S1) and $^{1}H/^{1}H$ COSY spectra of **1** displayed two spin systems, one composed by aliphatic protons from H_3-1 through H_2-5, and another between the four olefinic protons (H-10 through H-13) and the aliphatic protons H_2-14 and H_3-15. The HMBC spectrum (Supplementary Figure S3) exhibited correlations from H_2-5 to C-6 (δ_C 200.9) and C-7 (δ_C 107.0) (Figure 2), deduced to belong to a furanone ring. The methoxyl group H_3-18, with a proton resonance in ^{1}H NMR at δ 3.81, displayed long-range heteronuclear correlations to the carbonyl C-17 (δ_C 164.3) of the esther group, confirming the presence of a methoxycarbonyl group in the molecule (Figure 1). The presence of a double bond between C-6 (δ_C 200.9) and C-7 (δ_C 107.0) in the furanone moiety was deduced on the basis of similar ^{13}C NMR chemical shifts with other members of the same structural class.[10] The methyl group H_3-16 and the methine H-10 from the second spin system chain also showed key heteronuclear long-range correlations (Figure 2), to C-9 and to the ketone C-8 (δ_C 200.7), deduced to be part of the furanone moiety. An *E* geometry was assigned to the $^{10}\Delta$ double bond based on the existence of a coupling constant of 15.4 Hz between H-10 and H-11, whereas a coupling constant of 10.6 Hz between H-12 and H-13 secured a *Z* configuration for the $^{12}\Delta$ olefin.

These data indicated that **1** has a structure similar to the structure of graminin A,[3] the only differences between both compounds being the absence of $^{4}\Delta$ insaturation in the structure of **1** and the geometry of the $^{12}\Delta$ double bond. The trivial name graminin B is therefore proposed for compound **1**. The absolute configuration at the only chiral center of the furanone ring was assumed to be *R* after comparison of the optical rotation value ($[\alpha]_D^{20}-96$) with those reported for the synthetic enantiomers (−)-gregatin A and (+)-aspertetronin A.[10]

Fundación MEDINA, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Granada, Spain
Correspondence: Dr C Almeida or Dr F Reyes, Fundación MEDINA, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Avenida del Conocimiento 3, Parque Tecnológico de Ciencias de la Salud, Granada E-18016, Spain.
E-mail: celsoguerreiro@gmail.com or fernando.reyes@medinaandalucia.es
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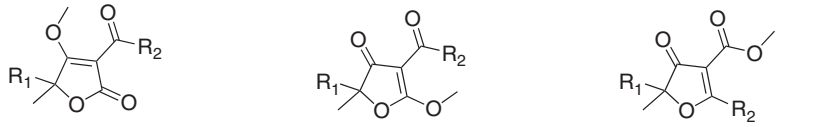

Figure 1 Structural revisions of natural furanones.

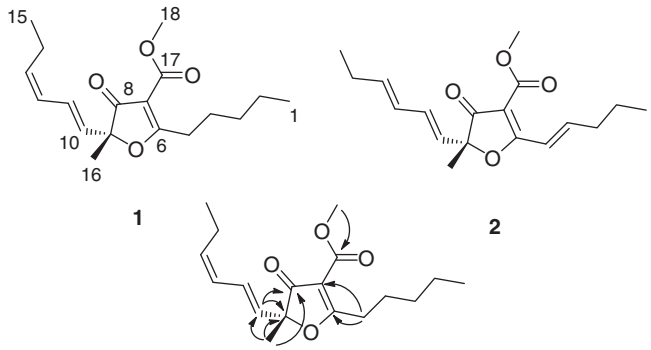

Figure 2 Structures of graminin B (**1**) and A (**2**) and key HMBC correlations observed for **1**.

Graminin B was tested in a panel of antimicrobial assays, namely against strains of *E. coli*, *A. baumannii*, *P. aeruginosa* and methicillin-resistant *S. aureus*.[11] The compound showed weak activity against the strain of *E. coli* (MB5746, EnvA/TolC) with an MIC of 64 μg ml^{-1} and a 76% growth inhibition at 21 μg ml^{-1}. We also observed some antibiosis against methicillin-resistant *S. aureus*, with a 77% growth inhibition when tested at 64 μg ml^{-1} (Supplementary Figure S9). No activity was detected against *A. baumannii* and *P. aeruginosa* at the highest concentration tested. Compound 1 was also inactive at a concentration of 64 μg ml^{-1} when tested in antifungal assays against *A. fumigatus* and *C. albicans*,[11] and in an assay to detect potentiators of the antifungal effect of caspofungin.[12] Additionally, graminin B was tested against three cancer mammalian cell lines, namely MiaPaca_2 (pancreas), HepG2 (liver) and MCF7 (breast), and displayed no activity at 20 μg ml^{-1}.[11]

To the best of our knowledge, *Panaconiothyrium* sp. is the fourth genus where furanone derivatives of this class have been found. As these compounds are known phytotoxins of important crops like wheat,[2,13] it is relevant to increase the awareness of the taxonomical spread of these phytotoxin-producing fungi and their presence on wheat, adzuki-bean and mung-bean crops.

EXPERIMENTAL PROCEDURE

General experimental procedure

Optical rotations were measured with a Jasco P-2000 polarimeter (JASCO Corporation, Tokyo, Japan). IR spectra were measured with a JASCO FT/IR-4100 spectrometer (JASCO Corporation). NMR spectra were recorded on a Bruker Avance III spectrometer (Bruker Biospin, Fällanden, Switzerland) (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm cryoprobe, using the signals of the residual solvent as internal references (δ_H 3.31 and δ_C 49.0 p.p.m. for CD_3OD). LC-UV-MS analysis was performed on an Agilent 1100 (Agilent Tehcnologies, Santa Clara, CA, USA) single quadrupole LC-MS system. HRESIMS and MS/MS spectra were acquired using a Bruker maXis QTOF (Bruker Daltonik GmbH, Bremen, Germany) mass spectrometer coupled to an Agilent 1200 LC (Agilent Technologies, Waldbronn, Germany). Acetone used for extraction was analytical grade. Solvents employed for isolation were HPLC grade.

Table 1 NMR spectroscopic data (500 MHz, CD_3OD) for Compound 1

Position	δ_c, *type*	δ_c, *(J in Hz)*	*COSY*	*HMBC (H to C)*
1	14.2, CH_3	0.94, t (7.0)	2	2, 3
2	23.3, CH_2	1.42, m	1, 3	1, 3,4
3	32.5, CH_2	1.42, m	2, 4	1, 2, 4, 5
4	27.3, CH_2	1.78, m	3, 5a, 5b	2, 3, 5, 6
5	31.8, CH_2	3.18, dt (14.0, 7.6)	5b, 4	3, 4, 6, 7
		3.00, dt (14.0, 7.4)	5a, 4	3, 4, 6, 7
6	200.9, C			
7	107.0, C			
8	200.7, C			
9	92.6, C			
10	128.8, CH	5.66, d (15.4)	11, 12	8, 9, 11, 16
11	127.7, CH	6.63, ddd (15.4, 11.0, 1.0)	10, 12	9, 12, 13
12	127.4, CH	6.0, t (10.9)	11, 13, 14	10, 14, 15
13	137.7, CH	5.57, dt (10.6, 7.7)	12, 14	9, 11, 14, 15
14	22.1, CH_2	2.21, m	12, 13, 15	12, 13, 15
15	14.4, CH_3	1.01, t (7.5)	14	13, 14
16	22.5, CH_3	1.54, s		8, 9, 10
17	164.3, C			
18	51.8, CH_3	3.80, s		17

Producing fungus and its characterization

The producing fungus (CF-217411) was isolated from aerial stems of *Laguncularia racemosa* (Combretaceae) collected at San Lorenzo National Park, Panama. Frozen stock cultures in 10% glycerol (−80 °C) are maintained in the collection of Fundación MEDINA. Total genomic DNA was extracted from mycelia grown on yeast mold agar.[14] The sequence of the ribosomal internal transcribed spacers and 5.8S gene and the initial 650 nt of the 5′end of the large RNA gene (D1 and D2 regions) were amplified by PCR, and sequenced, bidirectionally with the same primers used for PCR reactions, following standard procedures. Partial contigs obtained assembled with GeneStudio software (GeneStudio Inc., Suwanee, GA, USA). The sequences of the complete ITS1-5.8S-ITS2-28S region or independent ITS and 28S rDNA sequences were compared with GenBank or the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/) databases using BLAST. Species and genus groups were tested with Bayesian analysis employing the Markov chain Monte Carlo approach using MrBayes 3.01 (http://mrbayes.sourceforge.net/).[15]

Fermentation

Paraconiothyrium sp. (CF-217411, Supplementary Figures S7 and S8) was fermented by inoculating ten mycelia agar plugs into SMYA medium (bacto neopeptone 10 g, maltose 40 g, yeast extract 10 g, agar 3 g, and H_2O 1 l) into a flask (50 ml medium in a 250 ml Erlenmeyer flask). The flask was incubated on a rotary shaker at 220 r.p.m. at 22 °C with 70% relative humidity. After growing the inoculum for 7 days, a 3-ml aliquot was used to inoculate each

flask of the production medium STP (sucrose 75 g, tomato paste 10 g, malt extract 5 g, soy flour 1 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 9 g and H_2O 1 l). The flasks (100 ml medium per 500 ml Erlenmeyer flask) were incubated at 22 °C with 70% relative humidity on a rotary shaker at 220 r.p.m. for 21 days.

Extraction and isolation

The scaled-up fermentation broth (1 l) was extracted with acetone (1 l) under continuous shaking at 220 r.p.m. for 1 h. The biomass was then separated by centrifugation, and the supernatant (*ca* 2 l) was concentrated to 1 l under a stream of nitrogen. After concentration we noticed that the extract had precipitated. The precipitate was centrifuged and the pellet was considered as fraction 1. The supernatant solution was loaded (with continuous 1:1 water dilution, discarding the flow through) on a column packed with SP207SS (Sorbtech, Atlanta, GA, USA) reversed-phase resin (brominated styrenic polymer, 65 g) previously equilibrated with water. The loaded column was further washed with water (1 l) and afterwards eluted at 8 ml min^{-1} using a linear gradient from 10 to 100% acetone in water (in 12.5 min) with a final 100% acetone step (for 15 min), collecting 9 fractions of 20 ml, hence collecting in total 10 fractions, accounting with the first fraction of precipitated extract. Fractions were concentrated to dryness on a centrifugal evaporator. Bioassays revealed that fractions 1 (precipitate), 8 and 9 had relevant biological activity. Fraction 1 (precipitate) was selected for further fractionation by reversed-phase preparative HPLC (Agilent Zorbax SB-C_8 (Agilent Technologies, Santa Clara, CA, USA), 21.2 × 250 mm, 7 µm; 20 ml min^{-1}, UV detection at 210 nm) with an isocratic solvent system of 65/35 acetonitrile/water. Subfraction 5 of 5 was selected for purification by semi-preparative HPLC (Agilent Zorbax RX-C_8, 9.4 × 250 mm, 5 µm, 3.6 ml min^{-1}, UV detection at 210 nm) with an isocratic solvent system of 65/35 acetronitrile/water to yield **1** (1.0 mg, Rt 26 min).

Graminin B (**1**): colorless oil; $[\alpha]_D^{20}$−96.0 (*c* 0.06, MeOH),; UV λ_{max} (nm) 218 (sh), 239.5, 271 (sh) (Supplementary Figure S5); IR (ATR) ν_{max} 2959, 2932, 2872, 1746 (sh), 1705, 1579, 1437, 1387, 1199 cm^{-1} (Supplementary Figure S6); ^1H NMR and ^{13}C NMR, see Table 1. HRESIMS: *m*/*z*: calcd for $C_{18}H_{27}O_4$: 307.1904 $[M+H]^+$, found: 307.1906.

Biological activity

Antibacterial growth inhibition against *E. coli* (MB5746, EnvA/TolC), *E. coli* (MB2884), *A. baumannii* (MB5973), *P. aeruginosa* (PA01) and methicillin-resistant *S. aureus* MB5393 (Supplementary Table S1). Antifungal growth inhibition against *A. fumigatus* ATCC46645 and *C. albicans* MY0155. Potentiation of caspofungin fungal cell wall inhibition, and cytotoxicity against the human cell lines, MiaPaca_2 (pancreas), HepG2 (liver) and MCF7 (breast) were assayed as previously described.[11,12]

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)