Complete NMR Assignments of a new Prodigiosin isolated from *Streptomyces violaceusniger violaceusniger*

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Abstract: *A new prodigiosin 4'-methoxy-5'-[(1''-nonyl-5''-propyl-4''-cycle-1-penten-3''-imino)-methyl]-2,2'-bi-1H-pyrrole isolated for the first time from Streptomyces violaceusniger violaceusniger, was characterized by complete NMR assignments using 1D and 2D NMR techniques, including ^{1}H-^{15}N gHMBC and nuclear Overhauser enhancement experiments. The S. violaceusniger violaceusniger, an endophytic actinobacteria obtained from leaves of maize (Zea mays L.) which grown in several regions of the Sao Paulo State - Brazil, shown to be a strong potential inhibitor against several phytopathogens of economic interest such as: Fusarium moniliforme, Pythium aphanidermatum, Rhizoctonia solani, Sclerotinia sclerotiorum and Phytophthora parasitica.*

Introduction

Microbial biodiversity is very little known, mainly in specific habitats as the interior of plants and in extreme habitats. Related data are in general conservative and underestimated. However, the microorganisms are responsible for the output of hundreds of bio-pharmaceutical substances like, vaccines, enzymes, antibiotics, besides being a source of food and representing a market of dozens of billion dollars worldwide.[1]

Endophytic microorganisms, generally fungi and bacterias, live systematically in internal parts of healthy plants, without apparently causing problems to their hosts.[1]

The importance of actinobacteria has also been related with the output of several substances of pharmacological and industrial application. In agriculture, secondary metabolites produced by the microorganisms have been marketed like fungicides. Among the members of the order Actinomycetales, the Streptomyces genus is the one better studied biotechnologically. Species of Streptomyces produce antibiotics, enzymes and enzymatic inhibitors with applications in the areas of agriculture, human and veterinary medicine.[2]

Streptomyces violaceusniger violaceusniger, was isolated as an entophytic from leaves of maize (*Zea mays* L.), a plant cultivated annually all over the Brazilian territory, belonging to the family of the Gramineae, and utilised as an energy source in human and animal food. Recently the use of maize was extended by industrialisation, like the production of starch, alcohol, sweeteners, oils, etc.[3]

Antagonism tests carried out *in vitro* showed that actinobacteria has a strong inhibitory potential against several phytopatogens of economic interest such as: *Pythium aphanidermatum, Rhizoctonia solani Sclerotinia sclerotiorum, Phytophthora parasitica* and *Fusarium moniliforme*.[4]

In this study, metabolites produced by the actinobacteria were obtained through the

extraction of culture medium with ethyl acetate. The chemical composition of concentrated extracts was screened by ^{1}H Nuclear Magnetic Resonance (NMR), which showed signals in the region of N-H. After this preliminary analysis, the crude extract was purified by exclusion column chromatography (Sephadex LH-20). Fraction analyses by ^{1}H NMR, directed the isolation of an alkaloid of the prodigiosin class from fraction 42.

Nuclear Magnetic Resonance (NMR)[5] spectroscopy is one the most important instrumental analysis methods for natural products.[6] Routine one-dimensional (1D)[7] and two-dimensional (2D)[8] methods could be used to determine the complex structure of a compound by both an improvement to the hardware and the development of multi-pulse sequences.[9]

Prodiginines (prodigiosin-like) are a large family of pigmented oligopyrrole antibiotics with high potential medicinal properties as immunosuppressants and anti tumour agents that are produced by several actinomycetes and other eubacteria.[10-13] Although also active against bacteria, protozoa and pathogenic fungi the antibiotics are not utilised due to their toxicity.[14]

The main objective of this study was to analyse, by NMR, the chemical structure of the new prodigiosin.

Experimental

Collection of materials - The microorganism used in this study (*Streptomyces violaceusniger violaceusniger*) was part of the germ-plasma bank in the Laboratory of Environmental Microbiology of the EMBRAPA Environment – Jaguariúna-SP. This strain of actinobacteria showed a high inhibitory potential against the phytopathogenic fungi *Pythium aphanidermatum, Rhizoctonia solani Sclerotinia sclerotiorum, Phytophthora parasitica* and *Fusarium moniliforme*.[4]

Isolation of compounds - A strain of *Streptomyces violaceusniger violaceusniger* was grown for 7 days at 301 K in a flask containing 500 mL of PDA culture medium (Potato, Dextrose, Agar). After this period, culture extraction was performed by addition of 150 mL of ethyl acetate. The mixed flask contents were filtered, the solvent was removed under reduced pressure, yielding an extract (99 mg), which was further dissolved in MeOH (100%) and subjected to chromatography on a Sephadex LH-20 column (50 cm x 3,5 cm). MeOH (100%) was used for the isocratic elution. Fraction number 42 (5 mg) gave a dark violet solid identified as 4'-methoxy-5'-[(1''-nonyl-5''-propyl-4''-cycle-1-penten-3''-imino)-methyl]-2,2'-bi-1H-pyrrole by NMR spectroscopic analysis.

NMR analyses - All NMR spectra were recorded at 298 K on a Bruker DRX400 spectrometer operating at 9.4 T, observing ^{1}H at 400.13, ^{13}C at 100.61 and ^{15}N at 40.54 MHz, and using 5 mm direct and inverse probes. Prodigiosin was dissolved in 0.6 mL of $CDCl_3$ and transferred to a 5 mm NMR tube, tetramethylsilane (TMS) was the internal reference for chemical shifts (δ 0.0).

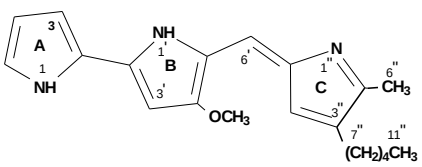
One dimensional ^{1}H, ^{13}C and DEPT 135 NMR spectra were acquired using standard pulse sequences, with spectral widths of 6410 Hz, 27027 Hz and 31847 Hz, respectively; 64k, 32k and 32k data points, respectively; pulse widths of 8.5 μs, 6.0 μs and 6.0 μs, respectively; relaxation delay of 1.2 s, 0.1 s and 1.0 s, respectively; acquisition time of 5.1 s, 0.6 s and 0.5 s, respectively; and 32, 48604

and 25360 scans, respectively. Spectra were processed using a Fourier transform with zero-filling with 64k, 32k and 32k, respectively, and by an exponential multiplication of the FIDs by a factor of 0.3, 3 and 3 Hz, respectively.

Standard pulse sequences were used for 2D shift correlation spectra. ^{1}H-^{1}H COSY experiment: spectral width of 6410 Hz in both dimensions, 4k x 203 data matrix, 16 scans per t_1 increment, acquisition time of 0.3 s and relaxation delay of 2.0 s. One-bond ^{1}H-^{13}C HSQC experiment: evolution delay of 1.7 ms for ^{1}J(C,H) of 145 Hz, 4k x 256 data matrix, 44 scans per t_1 increment, spectral widths of 6410 Hz in f_2 and 25156 Hz in f_1, acquisition time of 0.3 s and relaxation of delay 1.2 s. The long-range ^{1}H-^{13}C and ^{1}H-^{15}N HMBC experiments: evolution times of 62.5 ms for ^{LR}J(C,H) and ^{LR}J(N,H) for coupling constants of 8 Hz. The ^{LR}J(C,H) experiment: 2k x 121 data matrix, 80 scans per t_1 increment, spectral widths of 6410 Hz in f_2 and 25156 Hz in f_1, acquisition time 0.3 s and relaxation delay 1.2 s. The ^{LR}J(N,H) experiment: 2 k x 256 data matrix, 190 scans per t_1 increment, spectral widths of 6868 Hz in f_2 and 24330 Hz in f_1, acquisition time of 0.2 s and relaxation delay of 2.0 s. Spectra were processed by a Fourier transform using squared sine apodization on both dimensions and zero-filled to 2k x 1k data points in f_2 and f_1, respectively. The 1D nuclear Overhauser effect (n.O.e) spectra obtained by selective excitation and gradient selection were acquired using the double pulsed field gradient spin-echo (DPFGSE)-NOE[15] experiment, constant mixing time of 500 ms, relaxation delay of 2.0 s, 512 scans, spectral width of 6313 Hz and 32k data point. Spectra were processed with zero-filling to 32k data points and 1 Hz of line broadening.

Results and Discussion

The compound 4'-methoxy-5'-[(1''-nonyl-5''-propyl-4''-cycle-1-penten-3''-imino)-methyl]-2,2'-bi-1*H*-pyrrole (Figure 1) was obtained from ethyl acetate extract of the biomass produced by *Streptomyces violaceusniger violaceusniger* in PDA culture medium. This substance was obtained as a dark violet solid.



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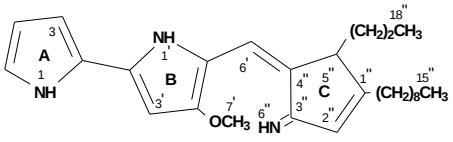

B

Figure 1. (**A**) prodigionin struture (SONG *et al,* 2006), (**B**) Prodigiosin isolated from the biomass produced by *Streptomyces violaceusniger violaceusniger* in PDA culture medium

All assignments of the ^{1}H, ^{13}C and ^{15}N chemical shifts, coupling constants and n.O.e enhancement observed in 1D (^{1}H, ^{13}C, DEPT135 and DPFGSE - *g*NOESY) and 2D (^{1}H-^{1}H *g*COSY-45, ^{1}H-^{13}C *g*HSQC, ^{1}H-^{13}C *g*HMBC and ^{1}H-^{15}N *g*HMBC) experiments are shown in Table 1. They were compared with the literature.[16]

Table 1. ^{1}H, ^{13}C, DEPT135, *g*COSY, *g*HSQC and *g*HMBC NMR data for 4'-methoxy-5'-[(1"-nonyl-5"-propyl-4"-cycle-1-penten-3"-imino)-methyl]-2,2'-bi-1*H*-pyrrole

	^{1}H δ(multiplicity, *J* in Hz)	^{13}C/DEPT 135 δ	^{15}N δ	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{15}N HMBC	n.O.e at[a]
1	12.57 (*brs*)	-	-	3; 4; 5	-	-	-
2	-	121.3 (C)	-	-	-	-	-
3	6.28 (*ddd*, 2.3; 2.4; 3.9)	110.6 (CH)	-	1; 4; 5	5	-	-
4	6.86 (*ddd*, 1.4; 2.5; 3.8)	115.3 (CH)	-	1; 4	2	-	-
5	7.16 (*ddd,* 1.3 ; 2.7; .9)	125.8 (CH)	-	1; 4	2; 4	-	1; 4
1'	12.70 (*brs*)	-	133.6	3'	4'	-	-
2'	-	146.4 (C)	-	-	-	-	-
3'	6.03 (*d*, 1.9)	91.7 (CH)	-	1'	2'; 5'	1'	-
4'	-	164.7 (C)	-	-	-	-	-
5'	-	119.5 (C)	-	-	-	-	-
6'	6.99 (*s*)	112.3 (CH)	-	-	3"; 4'	1'; 6"	5"
7'	3.96 (*s*)	57.7 (OCH_3)	-	-	4'	-	8"
1"	-	153.2 (C)	-	-	-	-	-
2"	6.19 (*d,* 1.9)	111.4 (CH)	-	6"	1"; 3"; 4"	6"	-
3"	-	149.3 (C)	-	-	-	-	-
4"	-	124.9 (C)	-	-	-	-	-
5"	2.52 – 2.45 (*m*)	38.6 (CH)	-	16"; 16"b; 17"a; 17"b	-	-	6'; 18"
6"	12.51(*brs*)	**-**	157.6	2"	-		-
7"a	3.18 – 3.11(*m*)	27.9 (CH_2)	-	7"b; 8"	1"; 2"; 8"; 9"	-	-
7"b	2.72 – 2.66 (*m*)			7"a; 8"			
8"	1.74 - 1.68 (*m*)	25.7 (CH_2)	-	7"a; 7"b	1"; 7"; 10"	-	-
***9"**	1.16	26.3 (CH_2)	-	10"	10"; 11"	-	-
***10"**	0.82	24.5 (CH_2)	-	-	11"; 13"	-	-
***11"**	0.82	21.7 (CH_2)	-	10"	9"	-	-
***12"**	0.82	23.5 (CH_2)	-	11"		-	-
***13"**	1.19	28.7 (CH_2)	-	-	10"; 11"; 12"	-	-
***14"**	0.98	25.5 (CH_2)	-	-		-	-
15"	0.82 (*t*, 7.4)	13.1 (CH_3)	-	-	13"	-	-
***16"a**	1.65	33.4 (CH_2)	-	5", 17"a, 17"b	3"; 5"; 18"	-	-
***16"b**	1.35						
***17"a**	1.63	28.9 (CH_2)	-	18"	5"; 3"; 16"; 18"	-	-
***17"b**	1.55						
18"	0.82 (*t*, 7.4)	11.6(CH_3)	-	17"a; 17"b	5"; 17"	-	-

[a] 1D n.O.e by selective excitation of each hydrogen signal
* Multiplicities not observed
brs - broad singlet, d - doublet, m - multiplet, s - singlet, t – triplet

The ^{1}H NMR spectrum of fraction 42 (Figure 2) displayed three ^{1}H *broad singlets* of hydrogens linked to nitrogens at δ 12.70, 12.57 and 12.51 (H-1', H-1 and H-6", respectively).[16] The aromatic and olefinic region exhibited six different resonance groups, from which one was a *singlet* at δ 6.99, two were *doublets* at δ 6.19 (*J* = 1.9 Hz) and 6.03 (*J* = 1.9 Hz) and the

other three were exhibited as *ddd* signals at δ 7.16 (*J* = 1.3; 2.7 and 3.9 Hz), 6.86 (*J* = 1.4; 2.5 and 3.8 Hz) and 6.28 (*J* = 2.3; 2.4 and 3.9 Hz). Their small coupling constant (< 4 Hz) is characteristic of five member heterocyclic (e.g. pyrroles).[17]The aliphatic region exhibited a 3H *singlet* at δ 3.96 (OCH_3). In addition, two 1H *multiplets* at δ 3.18 – 3.11 and 2.72 – 2.66 were observed (CH_2), a 1H *multiplet* at δ 2.52 – 2.45, several *multiplets* of 20 hydrogens (10 CH_2) between δ 1.80 and ~ 0.95, who multiplicities weren't observed, six hydrogens observed under the two *triplets* methyl groups signal (CH_2), and one *triplet* of two terminal methyl groups were observed at δ 0.82 (*J* = 7.4 Hz), completing two chains, one of C_9H_{19} and the other C_3H_7.

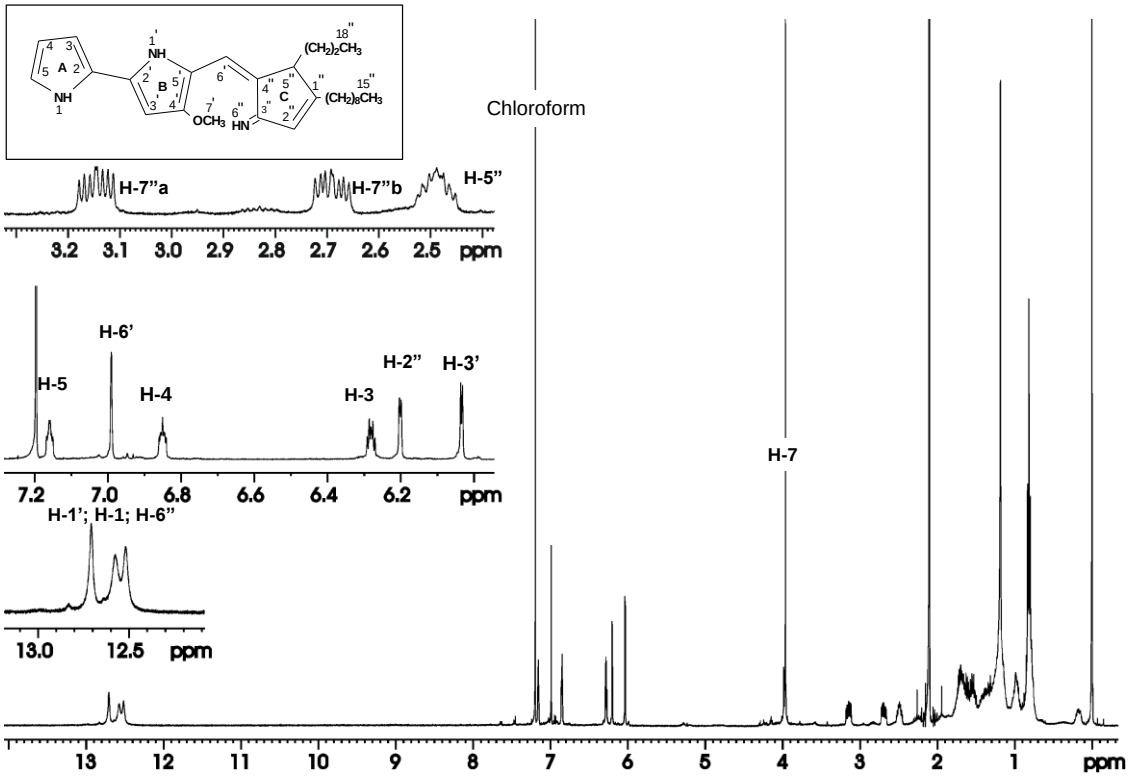

Figure 2. ^{1}H NMR spectrum of prodigiosin in $CDCl_3$.

The ^{13}C {^{1}H} NMR spectrum (Figure 3) displayed 27 carbon signals, including 3 sp^3, referring to two methyl and one methoxyl groups, 6 sp^2 carbons (5 aromatic and 1 olefinic carbons) and 1 CH of the imino ring (ring **C**). The 7 quaternary carbons (sp) were also observed in this spectrum.

The chemical shifts of carbons directly linked to hydrogens were attributed in accordance with the experiment ($^1J_{CH}$) ^{1}H-^{13}C *g*HSQC, as follows: δ 125.8 – C-5; δ 115.3 – C-4 and δ 110.6 – C-3 to pyrrolic ring **A**; δ 91.7 – C-3'; δ 57.7 – OMe to pyrrolic ring **B** (C-7'); δ 112.3 – C-6' (olefinic); δ 111.4 – C-2" and δ

38.6 – C-5" to ring **C**. The attribution of CH linked to H-5" (ring **C**) was only possible through the DEPT135 experiment. This CH differentiates the structure of prodigiosin from the structures of prodigiosins described in the literature.[16-18]

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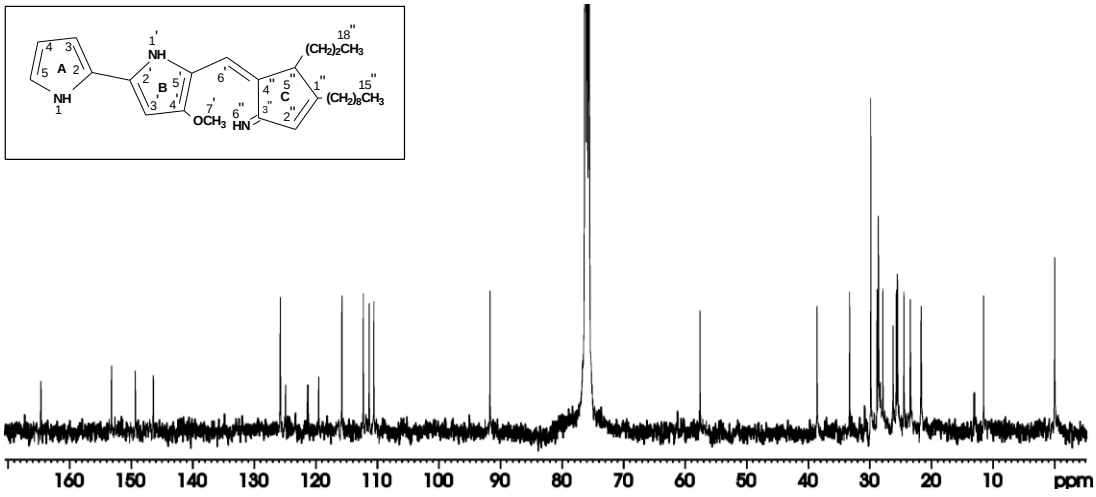

Figure 3. ^{13}C {^{1}H} NMR spectrum of prodigiosin in $CDCl_3$

In the ($^{3}J_{CH}$) ^{1}H-^{13}C *g*HMBC experiment (Figure 4), H-1', the hydrogen attached to nitrogen of ring **B,** presented correlation with C-4' (ring **B**) in δ 164.7. The aromatic hydrogens of ring **A** presented the following correlations: H-5 correlated with C-4 in δ 115.3 and C-2 in δ 121.3 (ring **A**). The H-3' (ring **B**) correlated with C-2' in δ 146.4 and C-5' in δ 119.5 (ring **B**). The olefinic hydrogen H-6' observed C-3" in δ 149.3 (ring **C**) and C-4' in δ 164.7 (ring **B**). The hydrogen H-2" (ring **C**) observed correlation with C-1" in δ 153.2, C-3" in δ 149.3 and C-4" in δ 124.9.

In the lateral chain C_9H_{19}, H-7"a and H7"b correlated with C-8" and C-9" in δ 25.7 and 26.3, respectively, and with C-2" and C-1" (ring **C**) in δ 111.4 and 153.2; H-8" correlated with C-7" in δ 27.9, C-10" in δ 24.5 and C-1" in δ 153.2; H-9" presented correlation with C-10" and C-11" in δ 24.5 and 21.7 (both of lateral chain); H-10" correlated with C-11" and C-13" in δ 21.7 and 28.9 H-11" correlated with C-9" in δ 26.3; H-13" correlated with C-10", C-11" and C-12" in δ 24.5, 21.7 and 23.5, respectively; H-15" (terminal methyl) correlated with C-13".

In the lateral chain C_3H_7, H-16"a and H-16"b correlated with C-18" (methyl) in δ 11.6, C-5" in δ 38.6, C-3" in δ 149; H-17"a and H-17"b

correlated with C-5", C-3"; C-16" and C-18". The H-18" presented correlation with C-5" and C-17" (in δ 28.9).

Through the ^{1}H-^{1}H *g*COSY experiment (Figure 5) the main homonuclear correlations can be observed. For ring **A**: H-5 (δ 7.16) correlated with H-1 (NH, in δ 12.57) and with H-4 (in δ 6.86); H-1 also correlated with H-5, H-4 and H-3 (in δ 6.28); H-3 correlated with H-4, H-5 and H-1. For ring **B**: H-3' (δ 6.03) correlated with H-1' (NH-1', δ 12.70). In ring **C**, H-2" (in δ 6.19) correlated with H-6" (NH-6", δ 12.51); H5" (in δ 2.48) showed correlation with H-16"a and H-16"b (δ 1.65 and 1.35) and with H-17"a and H-17"b (δ 1.63 and 1.55). Additionally, it was possible to attribute correlations in the lateral chains, H-16"a and H-16"b correlated with H-5"; H-17"a and H-17"b; the hydrogens H-17"a and H-17"b correlated with H-18" (methyl) in δ 0.82.

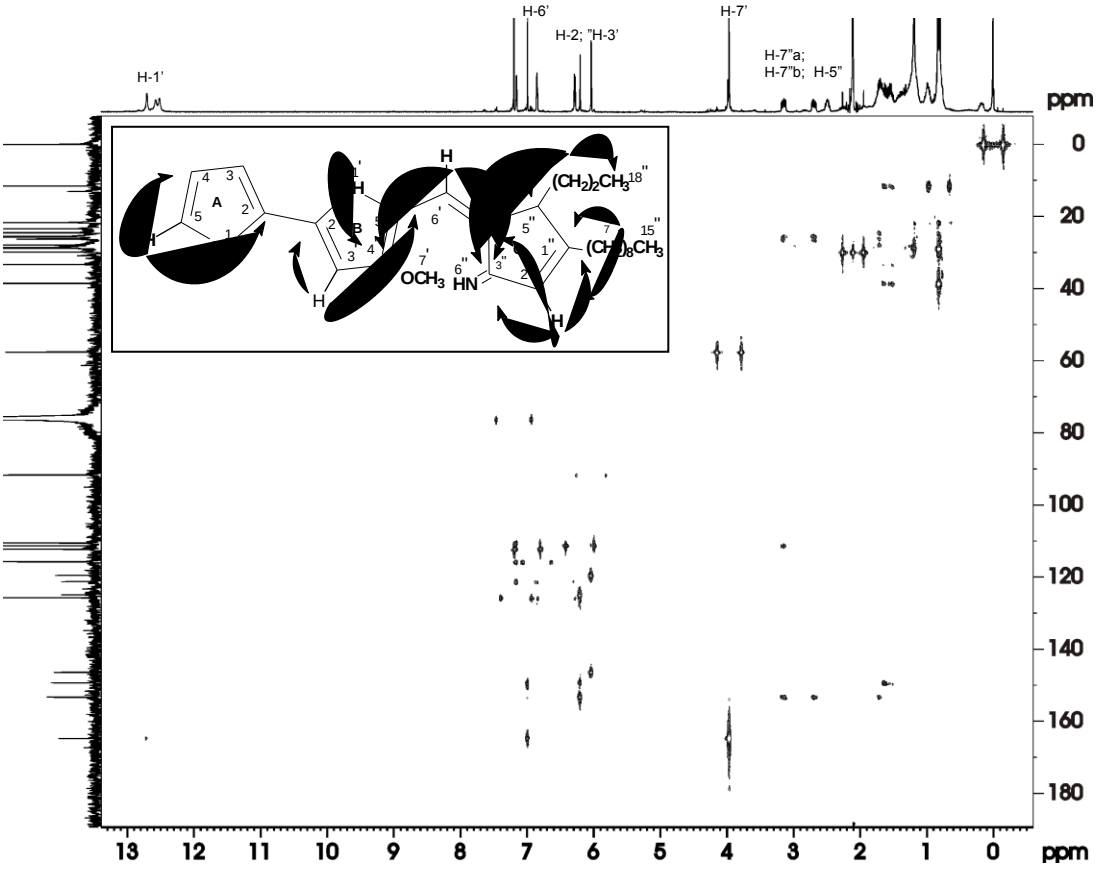

Figure 4. *g*HMBC NMR spectrum of prodigiosin in $CDCl_3$ and important long range correlations detected.

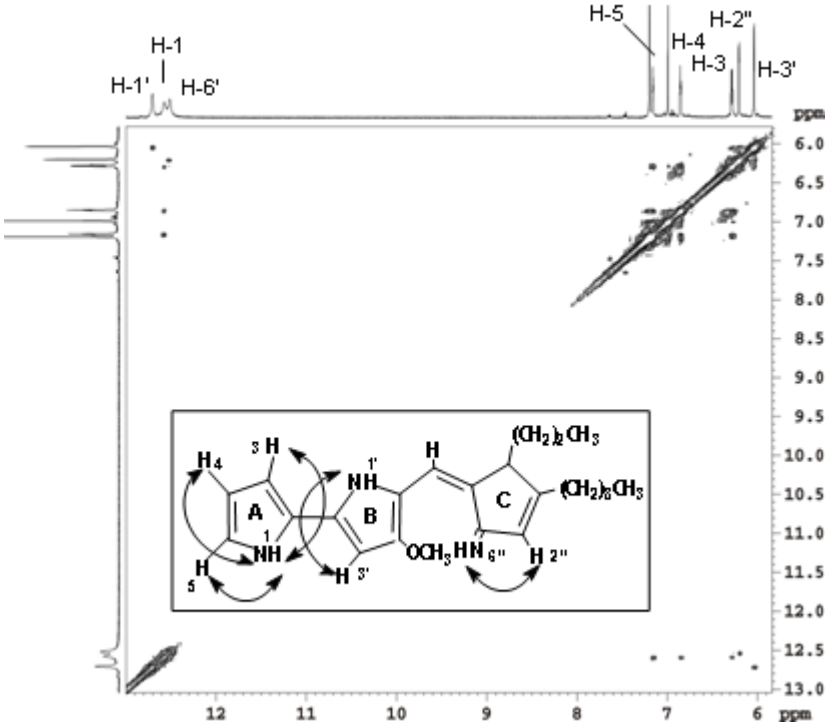

Figure 5. *g*COSY NMR spectrum of prodigiosin in $CDCl_3$ and important correlations detected

The lateral chain positions in ring **C**, as well as, the position of carbons linked to nitrogen in the same ring were confirmed by the ($^3J_{NH}$) ^{1}H-^{15}N *g*HMBC experiment (Figure 6), that showed four correlations: H-3' (ring **B**, δ 6.03) with N-1' in δ 133.6 (ring **B**); H-6' (δ 6.99) with N-1' in δ 133.6 and with N-6" in δ 157.6 (ring **C**); and H-2" (ring **C**, δ 6.19) correlated with N-6".

Additionally, H-5" and H-7" positions were confirmed through the *g*NOESY experiments (Figure 7). The irradiation in δ 2.52 – 2.45, H-5" (ring **C**), allowed to observe an n.O.e with the olefinic hydrogen H-6' (δ 6.99) and with methyl H-18" (δ 0.82), defining the C_3H_7 lateral chain position. When, the methoxy group in 3.96 (H-7', ring **B**) was irradiated, n.O.e was observed with H-8" (δ 1.74 – 1.68) of C_9H_{19} lateral chain. Also, the irradiation of H-6' (δ 6.99) presented n.O.e. with H-5", ring **C**, confirming the n.O.e when this was irradiated. When H-5 (δ 7.16, ring **A**) was irradiated, it caused n.O.e in H-4 (δ 6.86, ring **A**) and in H-1 (N-H, δ 15.57, ring **A**). The correlation of the H-5 with H-1 confirms the one observed in ^{1}H-^{1}H *g*COSY experiment. The results shows that the lateral chains and C-5" positions were only confirmed through the *g*COSY and nuclear Overhauser effect experiments.

The *g*NOESY and *g*COSY data complemented the ^{1}H-^{15}N *g*HMBC results, which did not present hydrogens correlating with nitrogen in ring **A**. The structure proposed based on the NMR experiments did not show alterations in ring **A**, and in comparison with the literature data[16] it was possible to suggest the structure for the compound.

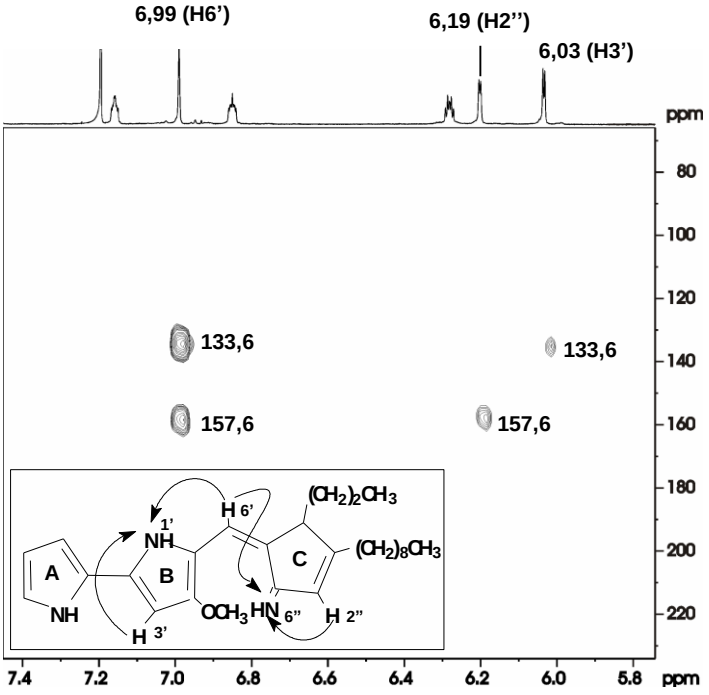

Figure 6: $^{1}Hx^{15}N$ *g*HMBC NMR spectrum of prodigiosin in $CDCl_3$ and important long range correlations detected.

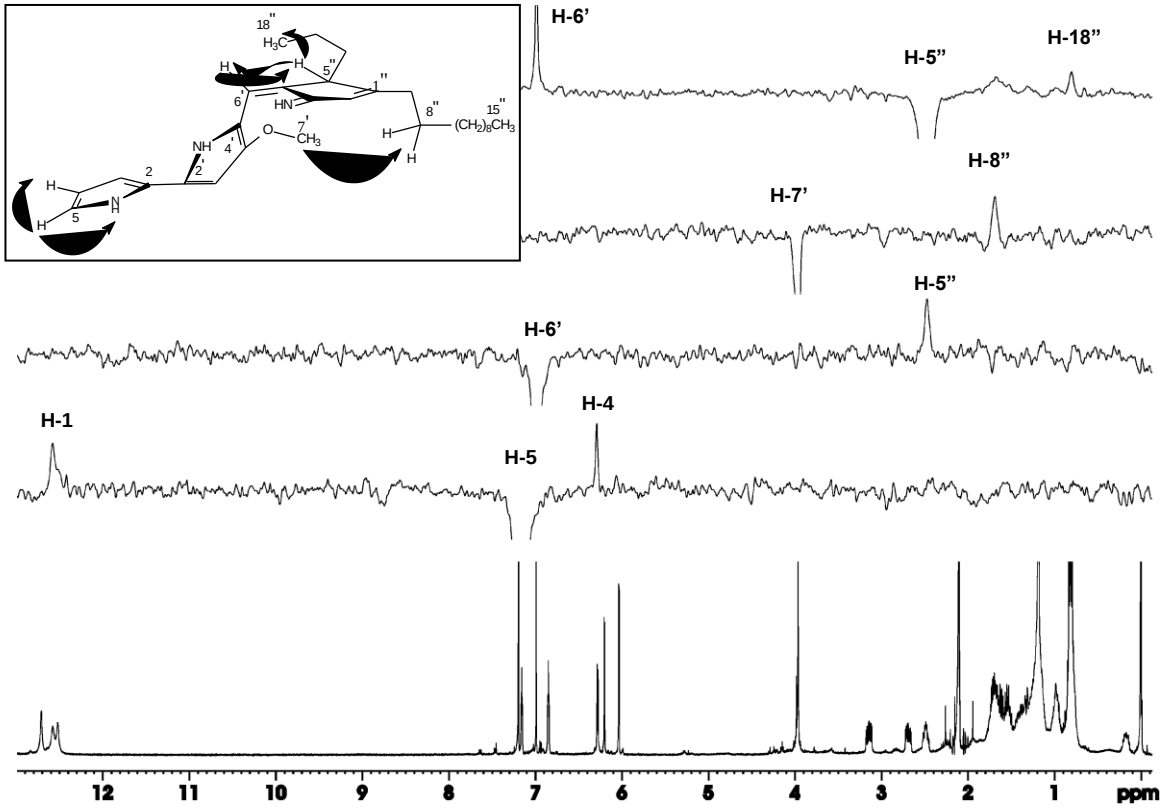

Figure 7. *g*NOESY NMR spectrum of prodigiosin in $CDCl_3$ and important spatial correlations detected.

The molecular weight of the compound was determined as 421.3049 by ESI-MSHR with positive ion mode. It had a $[421+H]^+$ ion at *m/z* 422.3049 in accordance with the $C_{27}H_{39}N_3O$ formula. In its MS/MSHR spectrum were observed the peaks at molecular ion *m/z* 422.2991, a base peak at *m/z* 407.2732 $[M+H-CH_3]^+$, a peak at *m/z* 392.2501 $[M+H-CH_2O]^+$ corresponding to the rearrangement of the hydrogen methyl group and the peak at *m/z* 268.1221, which was derived from the rearrangement in the ring **C** and loss of alkyl chains.

Conclusion

The isolation and characterisation of a new pink pigment produced by an actinobacteria (*Streptomyces violaceusniger violaceusniger*) were described. This is the first reported isolation of a prodigiosin with ring **C** modified. The methods applied (NMR and MS) provided unequivocal data allowing the complete structural elucidation from the prodigiosin. Moreover, antagonism tests carried out *in vitro* showed that the actinobacteria and metabolites had a strong inhibitory potential against several phytopatogens of economic interest.

Acknowledgments

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