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Unusual 2(1*H*)-Pyrazinones Isolated from a Culture of a Brazilian Marine-Derived *Streptomyces* sp.

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Four new secondary metabolites, giovaninones A-D (**1-4**), were isolated from an ethyl acetate extract of a culture of a marine-derived *Streptomyces* strain designated SS99BA-2. Chemical analysis was completely conducted in a coupled automated LC-SPE system with the use of a cryogenic NMR probehead and HRMS. The application of this system to identify, purify and elucidate all the structures is described.

Keywords: LC-SPE/NMR, Marine, *Streptomyces*, Secondary metabolite, 2(1*H*)-Pyrazinones.

The study of the chemistry of natural products derived from plants, animals, and microorganisms has resulted in the discovery of numerous organic compounds, many of which have applications such as pharmaceuticals, pesticides, antibiotics, and antifungals. The aquatic environment, especially oceans, which account for more than 95% of the Earth's biosphere, represents a source of potentially useful natural products, with more than 6000 new compounds having already been discovered. The target organisms include macroalgae, sponges, corals, ascidians, bryozoans, and mollusks, which are an excellent source of secondary metabolites [1].

Microorganisms are another possible source of natural products in the marine environment, since terrestrial microbes have already been shown to be able to produce useful organic compounds. Unlike plants and animals, microorganisms can be easily cultivated, and the processes can be readily scaled up [2]. This offers a promising renewable source of natural products and that is the reason why many compounds with interesting biological activities produced by such microorganisms have been described [3,4,5]. The bacterium designated as SS99BA-2 (GenBank AJ560629.1) is an isolated species of marine streptomycete found in samples of sediments collected at depths of between 12 and 15 m in the São Sebastião Channel (northern coast of São Paulo State, Brazil) [6]. Analysis of cell wall composition and the 16S rRNA sequence showed that the strain has 95% correlation with the 16S rRNA sequence of *Streptomyces violaceus*, and 95% correlation with *S. albofaciens* and *S. rimosus*. That work also described the structure of *N*-acetyl-γ-hydroxyvaline lactone, an unusual amino acid derivative isolated from the SS99BA-2 strain.

In the present investigation, SS99BA-2 was cultivated in shake flasks, and three main extracts were obtained by liquid-liquid fractionation. The ethyl acetate fraction exhibited activity against *Escherichia coli* and *Candida albicans* according to methodology published [7] and could, therefore, represent a source of potential candidates for new drugs. Analysis of this extract revealed the presence of four new metabolites, whose structures were fully elucidated by NMR spectroscopy and high-resolution mass spectrometry. The new secondary metabolites were isolated by using an automated coupled LC-SPE system, with monitoring of the UV-DAD response at 300 nm. The four compounds (segregated according to their chromatographic peaks) were trapped in polydivinylbenzene SPE cartridges during 20 sequential chromatographic runs. The compounds were subsequently eluted from the SPE cartridges with methanol-d_4 prior to NMR spectroscopic and high-resolution mass spectrometric analyses.

The molecular mass of **1** (238 Da), determined from the quasi-molecular ion [M-H] (*m/z* 237.0874) obtained by high-resolution mass spectrometry, was consistent with a molecular formula of $C_{11}H_{14}O_4N_2$. This requires six double bond equivalents, which, based on the carbon chemical shifts, were initially attributed to one carbonyl group, one carboxyl group, two C=C double bonds, one C=N double bond and one ring. The structure was assigned by analyses of the ^{1}H and ^{13}C one-dimensional spectra and ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC correlation maps. Two olefinic hydrogens (H-7 and H-8) are coupled to each other according to COSY, and both show HMBC connection to a carboxylic carbon (C-9, δ_C 170.8). In the one-dimensional ^{1}H spectrum, these hydrogen signals are both doublets (δ_H 6.72, d and δ_H 7.67, d), with a coupling constant of 15.8 Hz, consistent with the presence of an *E* double bond system, directly connected to the carboxyl group C-9 (δ_C 170.8). In the aliphatic region of the one-dimensional ^{1}H spectrum, a methyl group, which integrated for six hydrogen nuclei (δ_H 1.24, d), showed long distance correlations with C-3 (δ_C 163.8) and C-10 (δ_C 31.4), as shown in the ^{1}H-^{13}C HMBC experiment. Another aliphatic proton signal (δ_H 3.39, h), which integrated for one hydrogen nucleus, showed HMBC correlations to C-2 (δ_C 157.2), C-3 (δ_C 163.8), C-11 (δ_C 20.4), and C-12 (δ_C 20.4). Based on these data and the hydrogen resonance chemical shifts, as well as on the analysis of the HSQC information,

it was possible to conclude that this NMR data are consistent with an isopropyl group directly attached to an aromatic system. The signal at δ_H 3.39 was sufficiently deshielded to confirm this finding. An additional signal in the ^{1}H NMR spectrum was attributed to a hydroxyl methylene group. This signal integrated for two hydrogen nuclei (δ_H 4.60, s) and exhibited HMBC correlations to carbons C-3 (δ_C 163.8), C-4 (δ_C 138.8), C-5 (δ_C 126.4), C-6 (δ_C 157.2), C-7 (δ_C 138.7), and C-8 (δ_C 119.1). These multiple long distance correlations confirmed that this ^{1}H NMR signal was related to a -CH_2OH group directly connected to a cyclic aromatic system, as concluded previously.

Table 1: NMR spectroscopic data (^{13}C, 150MHz; ^{1}H, 600 MHz, MeOH-d_4) for giovaninone A (1).

no.	δ_C	δ_H (J in Hz)	HMBC[a]
2	157.2		
3	163.8		
5	138.8		
6	126.4		
6'	57.7	4.64, s	2, 3, 5, 6, 7, 8
7	138.7	7.67, d (15.8)	3, 5, 6, 8, 9
8	119.1	6.72, d (15.8)	5, 6, 7, 9
9	170.8		
10	31.4	3.39, h (7.0)	2, 3, 11, 12
11, 12	20.4	1.24, d (7.0)	3, 10

[a]HMBC correlations are from hydrogen(s) stated to the indicated carbon.

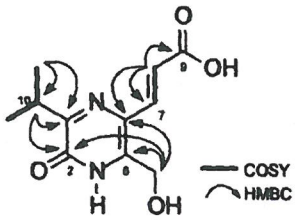
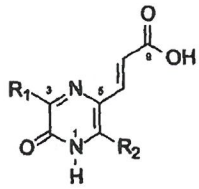


Figure 1: Key COSY and HMBC correlations and chemical structure of giovaninone A (1).

The ^{1}H NMR signal of H-7 also showed a large number of correlations, with C-3 (δ_C 163.8), C-5 (δ_C 138.8), C-6 (δ_C 126.4), C-8 (δ_C 119.1), and C-9 (δ_C 170.8). A correlation with C-2 (δ_C 157.2) was not observed, suggesting that C-2 is not close to this hydrogen but to the δ_H 4.64 hydrogen. A reasonable structure for all these data (Table 1) is consistent with a 3,5,6-trisubstituted 2(1*H*)-pyrazinone. Although the data collected with the use of NMR also suggest a possible 3,5,6-trisubstituted 4(1*H*)-pyridazinone system, a ^{1}H-^{15}N HMBC experiment revealed that the hydrogens H-7 and H-10 presented correlation with N-4 (δ_N 373.1) while H-6' presented correlation with N-1 (δ_N 357.9), leading to the 2(1*H*)-pyrazinone system. Figure 1 show the final structure for giovaninone A (1), as well as the main COSY and HMBC correlations that led to this conclusion.



(2) $R_1 = {}^{10}CH(CH_3)_2$ $R_2 = H$

(3) $R_1 = {}^{10}CH(CH_3)_2$ $R_2 = {}^{6'}CH_3$

(4) $R_1 = {}^{10}CH({}^{13}CH_3){}^{12}CH_2CH_3$ $R_2 = {}^{6'}CH_2OH$

Figure 2: Structures of compounds 2-4.

The other three compounds are structurally very similar to that of 1 (Figure 2). The mass of 2 (208 Da), determined from the value of the quasi-molecular ion [M-H] (*m/z* 207.0780), identified by HRMS, was consistent with the molecular formula $C_{10}H_{12}O_3N_2$. The masses of 3 (222 Da), obtained from [M-H] (*m/z* 221.0937), and 4 (252 Da), obtained from [M-H] (*m/z* 251.1043), were consistent with the formulae $C_{11}H_{14}O_3N_2$ and $C_{12}H_{16}O_4N_2$, respectively. All structures were confirmed by ^{1}H, ^{1}H-^{1}H COSY, HSQC, and HMBC NMR spectral data (Table 2).

Differences are only related to the R1 and R2 branches. The suggested structures seem to best fit the experimental data as a whole. When compared with 1, 2 has the same cyclic system, but possesses hydrogen in position 6 instead of the –CH_2OH group, as shown by the signal in the ^{1}H NMR spectrum (δ_H 7.38, s). This hydrogen is directly connected to carbon 6 (δ_C 126.7), as supported by the HSQC NMR data. Compound 3 also contains the same ring system, but there is a methyl group bonded to position 6. The ^{1}H NMR spectrum showed a new signal in the aliphatic region, integrating for three hydrogen nuclei in a methyl group (δ_H 2.36, s) directly attached to carbon C-6' (δ_C 14.9), as also supported by HSQC NMR data. In the case of 4, the difference lies in position 3, where there is a methyl-propyl group instead of the isopropyl group present in the other three compounds. This hypothesis is supported by all the signals observed in the aliphatic region. Although there is a stereogenic center in 4 (C-10), its stereochemistry was not possible to establish, due to the small amount of compound isolated. For the same reason, it was not possible to perform biological activity tests. To the best of our knowledge, all four structures are totally new, without described precedents. While for 1, 2, and 3 the presence of the isopropyl group suggests a biosynthetic pathway involving the amino acid *L*-valine, 4 was probably derived from a route involving *L*-isoleucine.

The structural feature of these new compounds is the presence of a completely substituted 2(1*H*)-pyrazinone ring skeleton, which is relatively rare in nature. It has been reported that arglecin, an antibiotic with the same ring system, can be produced by *Streptomyces toxytricini* (strain KA57-AG3) [8]. Another study, in which cultures of this microorganisms were supplied with *L*-arginine and *L*-leucine in a ^{14}C-labeling experiment showed

Table 2: NMR spectroscopic data (^{13}C, 150MHz; ^{1}H, 600 MHz, MeOH-d_4) for giovaninones B-D (2-4).

	giovaninone B			giovaninone C			giovaninone D		
no.	δ_C	δ_H (J in Hz)	HMBC[a]	δ_C	δ_H (J in Hz)	HMBC[a]	δ_C	δ_H (J in Hz)	HMBC[a]
2	157.1			157.5			157.4		
3	165.9			162.2			163.5		
5	130.3			137.5			139.0		
6	127.7	7.38, s	2, 3, 5, 7	127.2			127.8		
6'				14.9	2.36, s	2, 5, 6, 7	57.8	4.64, s	5, 6, 7
7	141.5	7.38, d (15.2)	3, 5, 8, 9	139.5	7.65, d (15.0)	3, 5, 6, 8, 9	138.9	7.60, d (15.0)	5, 6, 8, 9
8	118.5	6.66 d (15.2)	5, 6, 7, 9	118.4	6.68, d (15.0)	5, 6, 7, 9	119.6	6.74, d (15.0)	5, 6, 7, 9
9	170.8			171.0			171.1		
10	31.7	3.40, h (6.8)	2, 3, 11, 12	31.2	3.34, h (6.7)	3, 11, 12	37.8	3.23, q (6.8)	3, 11, 12, 13
11	20.3	1.24, d (6.8)	3, 10	20.4	1.23, d (6.7)	3, 10	28.6	1.55, ddq (6.8, 7.5, 13.5)	3, 10, 12, 13
11'							28.6	1.86, ddq (6.8, 7.5, 13.5)	3, 10, 12, 13
12	20.3	1.24, d (6.8)	3, 10	20.4	1.23, d (6.7)	3, 10	12.4	0.90, t (7.5)	10, 11, 13
13							12.6	1.21, d (6.8)	3, 10, 11, 12

[a]HMBC correlations are from hydrogen(s) stated to the indicated carbon.

that arglecin is biosynthetically produced by these two amino acids [9]. Argvalin was found as a new microbial metabolite related to arglecin by *Streptomyces filipensis* (strain KG62-AGl) [10, 11]. The metabolite is probably formed by a coupling between *L*-arginine and *L*-valine. Neither of these metabolites has a substituent on the C-5 position of the 2(1*H*)-pyrazinone system. Phevalin was also isolated as a new calpain inhibitor from a culture of *Streptomyces* sp. (strain SC433) [12]. This compound has a 2(1*H*)-pyrazinone system that is biosynthetically derived from a *L*-phenylalanine and *L*-valine coupling. Later, other researchers showed that these pyrazinone secondary metabolites are also produced by *Staphylococcus aureus*. The isolation of tyrvalin, phevalin and leuvalin was described [13]. Recently, two new 2(1*H*)-pyrazinones were isolated from a culture of *Streptomyces lucensis* [strain NBRC 13056T (AB184280)]: JBIR-56 and JBIR-57 [14]. The authors suggest that the unique structures of these new compounds may have been biosynthesized by a novel nonribosomal peptide synthetase. JBIR-56 and JBIR-57 consist of a unique skeleton connected to the peptide chain at the C-5 position of the pyrazinone ring.

When it comes to the compounds found in the present work, it seems that they follow the same amino acid coupling, but followed by condensation with malonyl-CoA at C-5 of the ring system. 2(1*H*)-pyrazinone is formed through the coupling between *L*-valine (A) and *L*-serine, leading to the intermediate B (Figure 3). The next step apparently involves a condensation of the intermediate B with malonyl-CoA leading to the intermediate C. A reduction in the carbonyl group of the side chain (C), followed by a loss of water, leads to giovaninone A (1). The others giovaninones would be formed according to the same biosynthetic pathway. Compound 2 is probably a coupling between *L*-valine and glycine, 3 is probably a coupling between *L*-valine and *L*-alanine, and 4 a coupling between *L*-isoleucin and *L*-serine. This intriguing biosynthetic origin is currently under investigation.

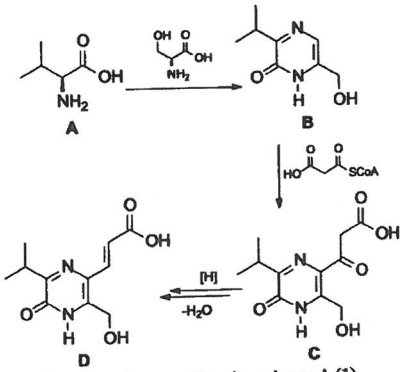


Figure 3: Biogenetic scheme proposed for giovaninone A (1).

Four new secondary metabolites were successfully isolated and identified through the use of LC-SPE/NMR hyphenated technology. Giovaninones A-D (1-4) were elucidated by extensive NMR and HRMS methods. Despite its rapidness in order to get information about the chemical composition, analytical separation is not convenient for isolating larger amounts of compounds. Therefore, a separation on a preparative scale must be conducted before extra experiments, such as stereochemistry determination and bioassays.

Experimental

General experimental procedures: The analyses employed a chromatograph (1200 series, Agilent GmbH) fitted with a G1311A quaternary pump, a G1322A degasser, a G1315D variable wavelength diode array detector, and a G1329A autosampler. Hystar 2.3 software (Bruker) was used to control the LC system. The chromatograph was directly coupled to an automatic cartridge exchanger (Bruker Biospin GmbH) equipped with a range of cartridges containing different stationary phases, to which the flow was directed automatically. The NMR measurements employed a Bruker Avance III instrument (14.1 Tesla / 600 MHz) equipped with an automatic sample changer and a TCI 5 mm triple resonance ($^{1}H/^{13}C/^{15}N$) z-field gradient cryo-probe. The probe was fitted with an automatic tuning and matching unit and a Cryo-FIT converter for coupling to the LC-SPE system. High-resolution mass spectrometry was carried out with an LTQ Orbitrap Velos FT-MS instrument (Thermo Fisher Scientific), equipped with an electrospray source (HESI-II) and operated in full scan negative and positive ionization mode.

Fermentation of* Streptomyces *sp. SS99BA-2 and workup: The marine *Streptomyces* sp. SS99BA-2 was cultivated in GYM culture medium (glucose 4 $g.L^{-1}$, malt extract 10 $g.L^{-1}$, yeast extract 4 $g.L^{-1}$, pH = 7.2). For the microorganism reactivation procedure, two 500 mL Erlenmeyer flasks containing 50 mL of reactivation medium were inoculated with the microorganism preserved in 3.5 mL CryoTube vials. The flasks were kept in a shaker at 200 rpm and a temperature of 28°C. After 24 h of cultivation, growth could be observed visually from the changes in turbidity and color of the culture broth, as well as the formation of a biofilm on the walls of the flasks. One of the Erlenmeyer flasks was then selected, in which the microorganism showed the best growth, by visual comparison of the amount of biofilm formed and the turbidity of the medium. The suspension in the chosen flask was used as inoculum for 4 500 mL Erlenmeyer flasks containing 40 mL of culture medium (GYM). These flasks were inoculated with 10 mL (20%) of suspension. This gave a 200 mL final volume of inoculum medium. Twenty Erlenmeyer flasks containing 40 mL of production culture medium (GYM) were inoculated with 10 mL of inoculum suspension, totaling a final volume of 1000 mL of production medium. The culture lasted 72 h. The resulting broth was centrifuged at 10,000 rpm for 20 min to separate the cells. The solution was extracted with ethyl acetate, and the solvent dried by speed-vacuum (Centrivap Concentrator, Labconco Corp.) to yield 10 mg of dry extract.

Isolation of four new dipeptide metabolites: A 3 mg portion of the dry ethyl acetate extract was dissolved in 1.5 mL of a 1:1 mixture of Milli-Q water and methanol. The solution was filtered through a PVDF membrane syringe filter (25 mm, 0.45 µm, Tedia Brazil) prior to HPLC analysis. The chromatographic separation was performed using a Eurobond Prontosil C18 column (125 x 4.0 mm, 5 µm). Gradient elution was performed using a combination of 0.05% TFA-HPLC (Tedia Brazil, lot # 1011342) in Milli-Q water (eluent A) and 0.05% TFA-HPLC (Tedia Brazil, lot # 1011342) in methanol (eluent B), with a linear gradient of 10% eluent B (held for 5 min) to 100% eluent B, over 45 min. The peaks of interest were detected using the UV response at 300 nm, and the corresponding compounds were adsorbed on solid phase extraction cartridges (HySphere Resin GP, 10 mm x 2 mm, 10 µm spherical polydivinylbenzene stationary phase) using an automatic cartridge exchanger (Bruker Biospin GmbH). Twenty consecutive chromatographic runs were performed, with 20 µL injections and a flow rate of 1.0 mL/min. After the adsorption process, the cartridges were dried with nitrogen for 30 min to remove residual solvent. Deuterated methanol-d_4 (99.8% D) was used to elute the compounds from the SPE cartridges directly into NMR tubes (Bruker, 3 mm o.d.).

Supplementary data: HPLC chromatogram of the ethyl acetate extract at 300 nm, ^{1}H NMR (600 MHz, CD_3OD), ^{13}C NMR (150 MHz, CD_3OD), COSY, HSQC and HMBC spectra of giovaninone A; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR spectra (150 MHz, CD_3OD) of giovaninone B; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR spectra (150 MHz, CD_3OD) of giovaninone C; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR spectra (150 MHz, CD_3OD) of giovaninone D. High resolution mass spectra of giovaninones A-D (**1-4**).

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