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Grividomycins I, II and III, New Antibiotics of the Streptogramin class from *Streptomyces* sp. HIL Y-8240155

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Abstract :

Three new antibiotics grividomycins I, II and III belonging to streptogramin class, have been isolated from a *Streptomyces* sp. HIL Y-8240155. The antibiotics are active against several resistant Gram positive bacteria. The structures of these compounds were elucidated mainly by HREIMS, GCMS and chiral GC.

Keywords : Antibiotics; Microorganism; Cyclic peptides

In the course of our screening for new antibiotics of the streptogramin class, active against normal and resistant Gram positive bacteria, three new antibiotics namely grividomycin I, II and III [1] were isolated from the fermentation broth of *Streptomyces* sp. HIL Y-8240155. Herein we report the fermentation, isolation, structure elucidation and biological properties of grividomycin I, II and III which are coproduced with neoviridogrisein II [2-5] and IV [6-7].

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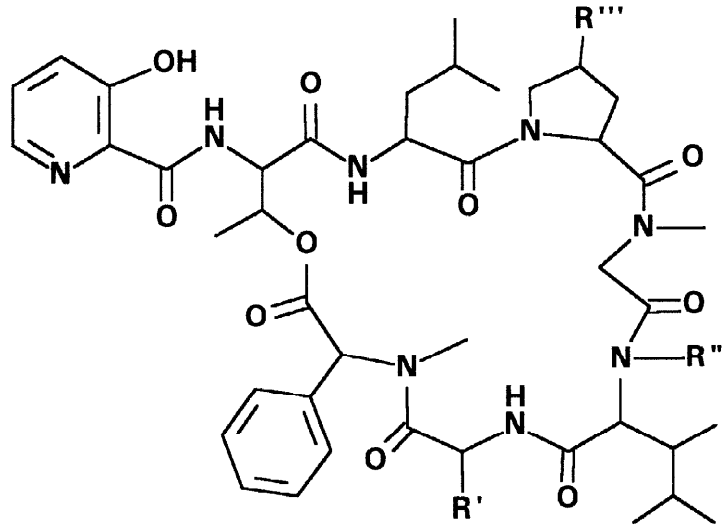
Results and Discussion

The physico-chemical properties of isolated neoviridogrisein IV were found to be identical to those reported in the literature [6]. The UV spectra of grividomycin I, II and III are identical to that of neoviridogrisein IV, suggesting the presence of 3-hydroxy picolinic acid moiety. This was also seen as a fluorescent spot in the TLC [silica gel/*n*-butanol:acetic acid:water(4:1:1)] of the hydrolysates (6N HCl/100^{0}C /17hours). The IR spectra of grividomycin I, II and III also indicate their similarity to neoviridogrisein IV. The ^{1}H NMR spectra of these compounds in DMSO-d_6 are rather complex due to slow equilibria amongst different conformers. This could be demonstrated by measurement of the spectra of grividomycin III at higher temperature.



		R'	R''	R'''
Grividomycin I	(1)	H	CH_3	OH
Grividomycin II	(2)	H	CH_3	OH
Grividomycin III	(3)	CH_3	H	OH
Neoviridogrisein IV	(4)	CH_3	CH_3	OH
Neoviridogrisein II	(5)	CH_3	CH_3	H

However the spectra were still too complex for a meaningful assignment of signals and were therefore of limited value for the purpose of structure elucidation.

Compounds belonging to this streptogramin type B class of antibiotics are depsipeptides and elucidation of the substructures of the compounds were achieved by analysis of the acid hydrolysates. Using neoviridogrisein IV as reference, the amino acid constituents in the total hydrolysates (6N HCl/100^{0}C /17hours) of grividomycin I, II and III were analysed by GCMS as

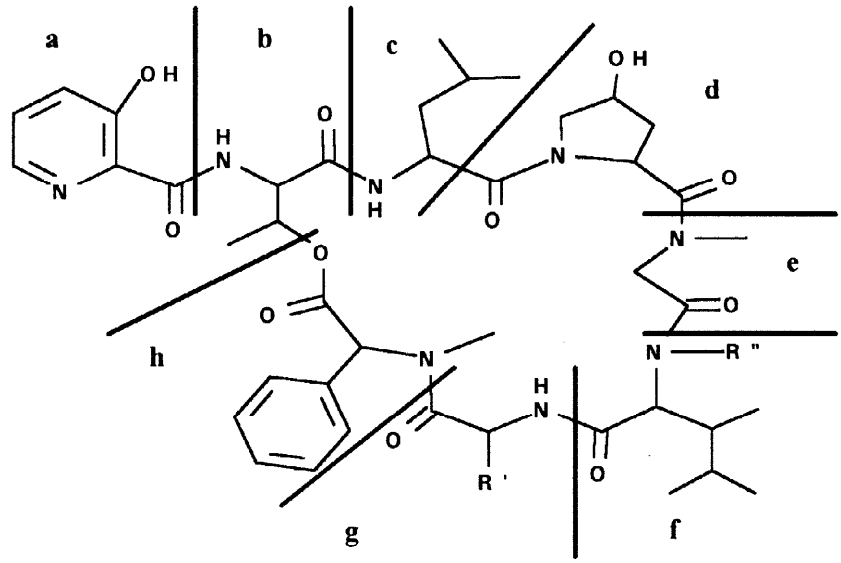
their N(O)-trifluoroacetyl methyl ester derivatives. 3-Hydroxy picolinic acid could not be unambiguously identified by this method. The amino acids identified are listed in Table 1.

Table 1.
Amino acids composition of grividomycins I, II and III

Amino acid	Grivido I	Grivido II	Grivido III	NVG
3-Hydroxy picolinic acid *	+	+	+	+
L-threonine	+	+	+	+
D-leucine	+	+	+	+
4-Hydroxy-D-proline	+	+	+	+
Sarcosine	+	+	+	+
N, β-dimethyl leucine	+	+	-	+
β-methyl leucine	-	-	+	-
L-Alanine	-	-	+	+
Glycine	+	+	-	-
Phenyl sarcosine	+	+	+	+

* by TLC and HREIMS

The compounds gave good EIMS. Again using the known structure of neoviridogrisein IV as reference and with the aid of exstensive high resolution mass measurements, the majority of the structure specific fragments could be assigned unequivocally to allow a reliable determination of the amino acid sequence. Summary of the mass values obtained for the appropriate elemental formulae are given in Table 2. It is to be noted that these assignments are different from the published data [8] which are based on the interpretation of low resolution MS measurements.



a - $C_6H_4NO_2$ b - C_4H_6NO c - $C_6H_{11}NO$ d - $C_5H_7NO_2$
e - C_3H_5NO f - $C_7H_{12}NOR''$ g - C_2H_2NOR' h - $C_9H_9NO_2$

Table 2.

Mass values (deviation ± 10 ppm) of the fragment formulae of grividomycin I, II, III and neoviridogresein IV

Key Ions	(4)	(1)	(2)	(3)
	R', R''= CH_3	R'= H,R''=CH_3	R'= H,R''=CH_3	R'= CH_3, R''=H
$M^{+\cdot}$	878.454	864.438	864.438	864.438
M-H_20	860.443	846.428	846.428	846.428
M-C_3H_7	835.399	821.383	821.383	821.383
M-C_4H_8	822.391	808.375	808.375	808.375
M-C_5H_{10}	808.376	794.360	794.360	794.360
Subunits :				
a	122.024	122.024	122.024	122.024
b-CO *	56.050	56.050	56.050	56.050
c-CO+H *	86.097(40)#	86.097(58) #	86.097(67) #	86.097(50) #
d-CO+H *	86.061(60) #	86.061(94) #	86.061(82) #	86.061(70) #
e-CO+H *	44.050	44.050	44.050	44.050
f-CO+H *	114.128	114.128	114.128	100.113
g-CO+H *	44.050	n. det.	n. det.	44.050
h-CO_2+H	120.081	120.081	120.081	120.081
Sequence Ions :				
a→(b-H)	205.061	205.061	205.061	205.061
a→(b-H)→c	318.145	318.145	318.145	318.145
a→(b-H)→c→(d-H)	430.185	430.185	430.185	430.185
(h+H)→(a→b)→c	483.224	-	-	483.224
(h+H)→(a→b)→c→(d-CO-H_2O)	550.267	550.267	550.267	550.267
(g+H)→h → (a→b)→c→(d-CO-H_2O)	621.304	607.288	607.288	621.304
(g-NH)→h→(a→b)→c→d→c	722.327	708.312	708.312	722.327
(g+CO)→h (a→b)→c→d→e	765.333	751.318	751.318	-
(c+CO-H)→d→e	324.156	324.156	324.156	324.156
(c+CO-H)→d→e→f	465.271	465.271	465.271	451.256
(d+H)→e→f→g	397.245	383.229	383.229	383.229
e→f	212.152	212.152	212.152	198.137

* R-CH=NH^{+}-H/CH_3, # Since m/z 86 is resolved into a doublet, the two relative intensities are given in parenthesis.

As can be seen from the amino acid composition and the connectivities, grividomycin I and II are desmethyl neoviridogrisein IV at the alanine unit and they form a pair which have the same chemical constituent but must be having difference with respect to one of the subunit - which means they are a pair of diastereoisomers. This difference may be present in the amino acid subunit presumably at phenyl sarcosine or N,β-dimethyl leucine. Grividomycin III is desmethyl of neoviridogrisein IV with the methyl loss at N,β-dimethyl leucine unit.

GC analysis of the derivatised (perfluoropropanoylation and *n*-propyl esterification) [9] acid hydrolysate (6N HCl/100^0C/17hours) using a chiral stationary phase (chirasil-L-valine) showed the presence of L-threonine, D-leucine, 4-hydroxy-D-proline and sarcosine in grividomycin I, II,

III and neoviridogrisein IV. Additionally glycine and three unknown amino acids were observed in grividomycin I and II. Also grividomycin III and neoviridogrisein IV showed the presence of L-alanine and three unknown amino acids. This established the configuration of some of the amino acids. However the configuration of other unknown amino acids which must be any three of 3-hydroxy picolinic acid, N,β-dimethyl leucine, β-methyl leucine and phenyl sarcosine (identified separately by GCMS and/or HREIMS) remain uncertain due to lack of authentic samples.

The *in vitro* activity (MIC) of grividomycin I, II and III is shown in Table 3. The compounds exihibited moderate activity against Gram positive bacteria and mycoplasma. Componds were found to be not active against Gram negative and fungal test organisms.

Table 3.
In vitro activity of grividomycin I, II and III

Test Organism	MIC(μg/ml)		
	Grivido I	Grivido II	Grivido III
Staphylococcus aureus 209P	06.4	00.4	06.4
S. aureus R85 *	25.0	06.4	06.4
S. aureus R85/M **	>50.0	50.0	50.0
S. aureus 712 #	25.0	12.5	25.0
S. aureus 789 #	25.0	12.5	50.0
S. aureus 3066 #	25.0	03.2	03.2
Streptococcus faecalis Eder ##	>50.0	50.0	>50.0
Sarcina lutea	12.5	03.2	12.5
Micrococcus luteus	12.5	00.4	06.2
Bacillus subtilis	03.2	01.6	12.5

* Tetracycline resistant, ** Erythromycin resistant, # Methicillin resistant, ## Macrolide resistant

Experimental

General experimental procedures

Melting points are uncorrected. UV spectra were recorded on a UVIKON 810 double beam spectrophotometer. IR spectra were obtained on a Perkin-Elmer 157 spectrophotometer. Optical rotations were measured using a Rudolph autopol III polarimeter. MS spectra were recorded on a VG-ZAB SEQ spectrometer. NMR studies were carried out on Bruker AM 270 spectrometer.

Fermentation

Strain HIL Y-8240155 was isolated from a soil sample collected at Panchgani, Maharashtra, India. The strain was characterised as belonging to the order *Actinomycetales*, family *Streptomycetaceae* and genus *Streptomyces*. A loopful of mature slant culture of HIL Y-8240155 was inoculated into 500 ml Erlenmeyer flasks containing 100 ml each of seed medium consisting of glucose 1.5%, soyabean meal 1.5%, cornsteep liquor 0.5%, NaCl 0.5%, and $CaCO_3$ 0.2% (pH adjusted to 7.0 before autoclaving). The flasks were shaken on a rotary shaker at 240 rpm for 72 hours at 30°C. The resultant seed culture (2 litres) was inoculated into two 15-liter fermenters containing 10 liters of the above seed medium. The aeration and agitation of the fermentation were maintained at 7 lpm and 170 rpm respectively and the temperature at 28°C. The fermentation was carried out for 24 hours and the resultant seed culture was inoculated into a 390-liter fermenter containing 280 liters of production medium consisting of glucose 1.5%, soluble starch 2.0%, soyatone 0.3%, peptone 0.3%, $CaCO_3$ 0.2%, NaCl 0.2% , cornsteep liquor 0.2%, $(NH_4)_2SO_4$ 0.05%, Desmophen$^{(R)}$ 0.025% (pH 7.0 before autoclaving). The aeration and agitation were maintained at 170 lpm and 120 rpm respectively and the temperature at 28°C. The fermentation was carried out for 44 hours. The production of the antibiotic and its purification were monitored by activity against *Staphylococcus aureus* 209P.

Isolation

The culture broth (280 liters) was harvested and centrifuged to separate the mycelium. The culture filtrate (250 liters, pH 6.9) was extracted with ethyl acetate (125 liters) using a counter current extractor(Westphalia). The mycelial cake (16.5 kg) was extracted with 120 liters of acetone, and was concentrated under reduced pressure, diluted with 100 liters of water and extracted with ethyl acetate (120 liters) using the extractor. All the ethyl acetate extracts were combined and concentrated under reduced pressure to get 100 g of oily crude material. This material was repeatedly triturated with 2 liter portions of petroleum ether(60-80°C) to get solid material which was filtered and washed with 2.5 liters of petroleum ether(60-80°C) and dried to get 35 g of semipure material. This material was chromatographed using silica gel (60-120 mesh, 60x8 cms, flow rate 40 ml/ min.) using increasing amounts of methanol (1, 2, 2.5, 3, 3.5 and 4 %) in chloroform. The monitoring of the purification was done by bioactivity against *Staphylococcus aureus* 209P and TLC(silica gel/ 7%methanol in chloroform/uv 366 nm).

The active fractions from 1-2 % methanol on concentration gave 7 g of a material which mainly contained neoviridogrisein IV with small amounts of neoviridogrisein II . The active fractions from 2 -3 % methanol on concentration gave 16 g of a mixture which mainly contained neoviridogrisein IV and grividomycin I, II and III.

This 16g lot was flash chromatographed on silica gel (230-400 mesh, 250 g, flow rate 50 ml/min.) using chloroform and gradient of methanol in steps of 0.5% for elution. The active fractions from 1-2 % methanol on concentration gave mainly neoviridogrisein IV (7 g). Further, a mixture of grividomycin I, II and III eluted out with 2-3 % methanol which on concentration yielded 4 g of enriched material.

This was furthur chromatographed on silica gel (450-550 mesh, 250 g, flow rate 10 ml/ min.) using chloroform and gradient of methanol in steps of 0.25% for elution. Pure grividomycin I was eluted with 1% methanol in chloroform which on concentration yielded 40 mg of white amorphous powder. Further elution with 1.5% methanol in chloroform yielded 200 mg of white amorphous powder of pure grividomycin II. Continued elution with 2% methanol in chloroform yielded 250 mg of white amorphous powder of pure grividomycin III.

The combined samples enriched in neoviridogrisein IV (14 g) on furthur purification (silica gel column chromatography using chloroform-methanol gradient) yielded 10 g of pure compound neoviridogrisein IV.

Grividomycin I : White powder; m.p. 141-4^{0}C; soluble in chloroform, ethyl acetate, methanol and DMSO; $[\alpha]_D$ -21.9^0 (*c* 0.265, chloroform); UV (MeOH) 213, 303 nm, (+NaOH$^+$) 216, 248(sh), 338 nm; TLC Rf : 0.59 [silica gel / MeOH:$CHCl_3$ (7:93)], IR (KBr) cm^{-1} : 3500, 3000, 1740, 1650, 1500, 1300, 1260, 1240, 1090, 960; HREIMS : 864.438 (M^+).

Grividomycin II : White powder; m.p. 153-6^{0}C; soluble in chloroform, ethyl acetate, methanol and DMSO; $[\alpha]_D$ +17.1^0 (*c* 0.350, chloroform); UV (MeOH) : 210, 304 nm, (+NaOH$^+$) 214, 248(sh), 338 nm; TLC Rf : 0.48 [silica gel / MeOH:$CHCl_3$ (7:93)]; IR (KBr) cm^{-1} : 3500, 3000, 1740, 1650, 1500, 1455, 1300, 1200,1115, 1090, 1010; HREIMS : 864.438 (M^+).

Grividomycin III : White powder, m.p. 175-6^{0}C, soluble in chloroform, ethyl acetate, methanol and DMSO, $[\alpha]_D$ -49.8^0 (*c* 0.305, chloroform), UV (MeOH) 210, 304 nm, (+NaOH$^+$) 212, 248(sh), 338 nm, TLC Rf : 0.41 [silica gel / MeOH:$CHCl_3$(7:93)], IR (KBr) cm^{-1} : 3500, 3000, 1740, 1650, 1505, 1440, 1375, 1280, 1215, 1060, 980; HREIMS : 864.438 (M^+).

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