Val-Geninthiocin: Structure Elucidation and MS^n Fragmentation of Thiopeptide Antibiotics Produced by *Streptomyces* sp. RSF18

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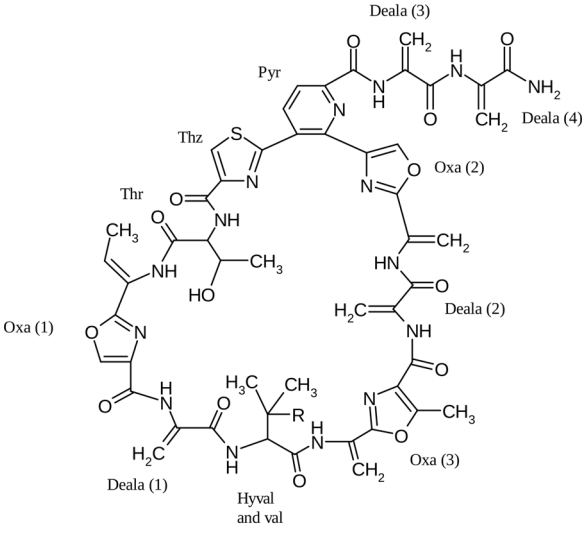
Val-Geninthiocin (**2**), a new member of thiopeptide antibiotics, was isolated from the mycelium of *Streptomyces* sp. RSF18, along with the closely related geninthiocin (**1**) and the macrolide, chalcomycin. By intensive NMR and MS studies, Val-geninthiocin (**2**) was identified as desoxygeninthiocin, a thiopeptide, containing several oxazole and thiazole units and a number of unusual amino acids. Compound **2** shows potent activity against Gram-positive bacteria and minor antifungal activity, while it is not effective against Gram-negative bacteria or microalgae. Here we describe the fermentation, isolation and structure elucidation as well as the biological activity of **2**.

Key words: Val-Geninthiocin, Thiopeptide Antibiotic, *Streptomyces*

Introduction

Thiopeptide antibiotics are forming a group of cyclic peptides characterized by several common structural features, such as oxazole and thiazole units and unusual amino acids; especially dehydroamino acids are typical [1, 2]. Thioxamycin [3], berninamycin A [4], sulfomycin I [5 – 7], and A10255 [8] *e. g*. possess the thiazole-pyridine-oxazole substructure, while thiocillin I [9, 10], micrococcin P [11] and GE2270 A [12] are characterized by a thiazole-pyridine-thiazole moiety. Thiopeptides have been used as antibacterial agents against Gram-positive bacteria and anaerobes, including pathogens resistant to antibiotics currently in use [13, 14], and also have potential as growth inhibitors of the human malaria parasite [15]. They were discovered as antibiotics in diverse bacteria including *Streptomyces*, *Bacillus*, and *Micrococcus* [16, 17]. Thiopeptides have been proved later as effective growth promoters for domestic animals [17, 18]. Most of the thiopeptide antibiotics inhibit protein synthesis in bacteria and share a common mode of action [19]. Thiostrepton, whose antibiotic activity is best understood, acts by binding tightly to the prokaryotic ribosome and thus inhibits translation [19, 20]. Geninthiocin (**1**) is known as an activating agent for transcription of the *tip A* promoter in streptomycetes [21].



1: Geninthiocin R = OH
2: Val-Geninthiocin R = H

In our search for new antibiotics from terrestrial bacteria, the crude extracts of *Streptomyces* sp. isolate RSF18 showed a potent activity against the Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Streptomyces viridochromogenes* (Tü 57), beside a weak antifungal activity against *Mucor miehei* (Tü 284) and *Candida albicans*. Extraction of the

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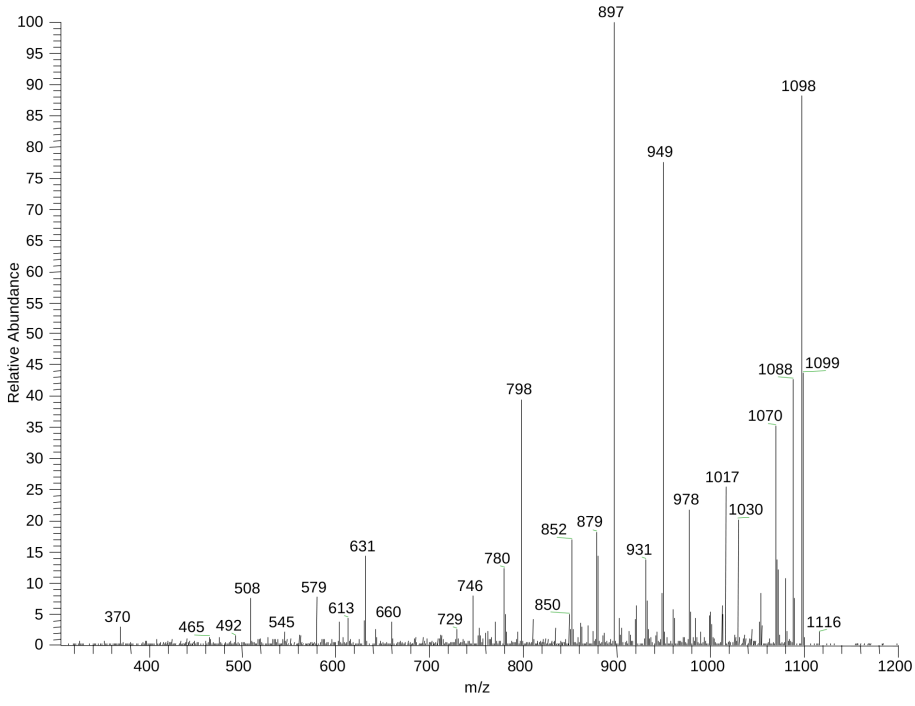

Fig. 1. CID-MS/MS spectrum of Val-geninthiocin (**2**); fragmentation of $[M+H]^+$ measured on a quadrupole ion trap instrument.

mycelia cake followed by a series of chromatographic steps afforded two thiopeptides, geninthiocin (**1**) and the new closely related Val-geninthiocin (**2**) as two colorless solids. Val-Geninthiocin (**2**), a desoxy-derivative of geninthiocin (**1**), is an UV absorbing middle-polar substance, which turned yellow by spraying with anisaldehyde/sulfuric acid reagent. Extraction of the filtrate afforded chalcomycin, a well-known macrolide antibiotic [22].

Results and Discussion

Well-grown agar plates of strain RSF18 were used to inoculate 60 of 1 L Erlenmeyer flasks, each containing 250 mL of M_2 medium. The fermentation was carried out at 95 rpm on a linear shaker for 7 d at 28 °C, forming a yellowish brown culture broth. After harvesting, the broth was filtered over Celite, and the filtrate was adsorbed on Amberlite XAD-16 resin, followed by elution with methanol. After concentration, the aqueous residue was further extracted with ethyl acetate followed by evaporation *in vacuo*, yielding 1.30 g of crude extract. The mycelial cake was extracted with ethyl acetate followed by acetone; evaporation and re-extraction with ethyl acetate delivered in a similar way 6.85 g crude extract. TLC of both extracts from water and mycelia, respectively, showed a completely different metabolic pattern. Work-up of the mycelial extract using a silica gel column and size exclusion chromatography resulted in geninthiocin (**1**; 78.6 mg) and a second closely related peptide (**2**; 27.5 mg), as the NMR data indicated. Separation of the filtrate extract delivered chalcomycin (19.8 mg) as a white solid.

HRMS of the main peptide isolated from *Streptomyces* sp. RSF18 delivered the formula $C_{50}H_{49}N_{15}O_{15}S$, while the minor component had the composition $C_{50}H_{49}N_{15}O_{14}S$. A data base search pointed to geninthiocin (**1**) for the former one, while the second product was new. As part of our ongoing structure elucidation of *cyclo*peptides by MS methods, it was of interest to investigate the fragmentation behavior of both metabolites in parallel. For this purpose, MS^2 and MS^3 experiments using a quadrupole ion trap were performed (Table 1), and additionally, high-resolution CID-MS/MS measurements were carried out on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer to determine the elemental composition of key fragments (Table 2).

Tandem mass spectrometry is a very powerful method for sequence analysis of peptides and proteins. The fragmentation of linear peptides was described

Table 1. MS^2 and MS^3 product ions of the $[M+H]^+$ precursor ions of geninthiocin (**1**) and Val-geninthiocin (**2**) obtained on a quadrupole ion trap mass spectrometer.

Geninthiocin (**1**)		Val-Geninthiocin (**2**)		
MS^2 fragment ion m/z	MS^3 fragment ions m/z	MS^2 fragment ion m/z	MS^3 fragment ions m/z	Proposed MS^2 fragmention
1115		1099		$-NH_3$
1114		1098		$-H_2O$
1104		1088		–CO
1074	1056; 1046; 988; 936; 907; 855; 812; 798			$-C_3H_6O$ from Hyval
1046	1028; 1018; 985; 949; 879; 851	1030	1012; 1002	–(H-Deala-NH_2)
1017	1000; 999; 982; 931; 879; 862; 798; 579	1017	1000; 999; 931; 879; 798; 579; 493	–Hyval/Val
994	966; 936; 974; 370	978	960; 950; 370	$-C_6H_6N_2O_2$ from side chain
965	948; 947; 921; 907; 903; 879; 850; 827; 798; 631	949	931; 921; 905; 903; 863; 850; 811; 631	–(H-Oxa-NH_2)
947		931		–(H-Oxa-NH_2)–H_2O
913	896; 895; 877; 855; 852; 837; 798	897	879; 851; 833; 811; 798; 793; 579	–Oxa–Deala
907	889; 879; 863; 821; 769; 752; 631			$-C_{10}H_{15}N_3O_3$–(H-Oxa-NH_2)–C_3H_6O
896		880		–H–Oxa–Deala–NH_2
895	878; 877; 851	879	861; 851	–Oxa–Deala–H_2O
889				$-C_{10}H_{17}N_3O_4$–(H-Oxa-NH_2)–C_3H_6O–H_2O
		852		
850		850		–H–Hyval/Val–Oxa–NH_2
798	781; 780; 770; 754; 712; 660; 631; 579	798	781; 780; 770; 754; 712; 660; 631; 579	–Hyval/Val–Oxa–Deala
780		780		–Hyval/Val–Oxa–Deala–H_2O
746	729; 728; 660; 608; 579	746	729; 728; 660; (608; 579)	–Deala–Oxa–Hyval/Val–$COCCH_2$ +2H
631	603; 545; 493	631	603; 545; 493	–H–Oxa–Deala–Hyval/Val–Oxa–NH_2
579	551; 535; 493; 441	579	561; 551; 535; 493; 441	–Oxa–Deala–Hyval/Val–Oxa–Deala
508	370	508	370	

Table 2. Confirmation of key fragments by exact mass determination using a FT-ICR mass spectrometer.

Mass (measd.)	Composition	Mass (calcd.)	Δm (ppm)	Fragmentation
Geninthiocin (**1**)				
965.2619	$C_{43}H_{41}N_{12}O_{13}S_1$	965.2631	1.3	$[M+H-C_7H_9N_3O_2]^+$
947.2523	$C_{43}H_{39}N_{12}O_{12}S_1$	947.2526	0.2	$[M+H-C_7H_{11}N_3O_3]^+$
907.2203	$C_{40}H_{35}N_{12}O_{12}S_1$	907.2213	1.1	$[M+H-C_{10}H_{15}N_3O_3]^+$
889.2102	$C_{40}H_{33}N_{12}O_{11}S_1$	889.2107	0.6	$[M+H-C_{10}H_{17}N_3O_4]^+$
798.2040	$C_{35}H_{32}N_{11}O_{10}S_1$	798.2049	1.1	$[M+H-C_{15}H_{18}N_4O_5]^+$
746.2095	$C_{32}H_{32}N_{11}O_9S_1$	746.2100	0.7	$[M+H-C_{18}H_{18}N_4O_6]^+$
631.1348	$C_{28}H_{23}N_8O_8S_1$	631.1354	0.9	$[M+H-C_{22}H_{27}N_7O_7]^+$
Val-Geninthiocin (**2**)				
949.2678	$C_{43}H_{41}N_{12}O_{12}S_1$	949.2682	0.4	$[M+H-C_7H_9N_3O_2]^+$
931.2577	$C_{43}H_{39}N_{12}O_{11}S_1$	931.2576	0.1	$[M+H-C_7H_{11}N_3O_3]^+$
897.2723	$C_{40}H_{41}N_{12}O_{11}S_1$	897.2733	1.1	$[M+H-C_{10}H_9N_3O_3]^+$
798.2036	$C_{35}H_{32}N_{11}O_{10}S_1$	798.2049	1.6	$[M+H-C_{15}H_{18}N_4O_5]^+$
631.1359	$C_{28}H_{23}N_8O_8S_1$	631.1354	0.8	$[M+H-C_{22}H_{27}N_7O_6]^+$

comprehensively, and detailed knowledge of the fragmentation mechanism has been obtained [23, 24]. However, the structure analysis of cyclic peptides, which represent an important class of bioactive natural products, by mass spectrometry remains a challenging task. Complex fragmentation patterns by two-bond cleavage at different ring positions, ring-opening reactions and uncommon rearrangement reactions complicate the interpretation of CID-MS/MS spectra [25, 26]. Frequently, higher-order MS^n investigations are required. Furthermore, cyclic peptides produced as secondary metabolites by bacteria often contain uncommon amino acid residues leading to different fragmentation reactions.

In general, for sequencing linear peptides, fragmentation of the $[M+2H]^{2+}$ ions is used successfully, but in the case of geninthiocin (**1**) and its minor congener, fragmentation spectra of those ions were dominated by doubly charged fragment ions without significant sequence information. Furthermore, $[M+2H]^{2+}$ ions were observed with very low intensity under

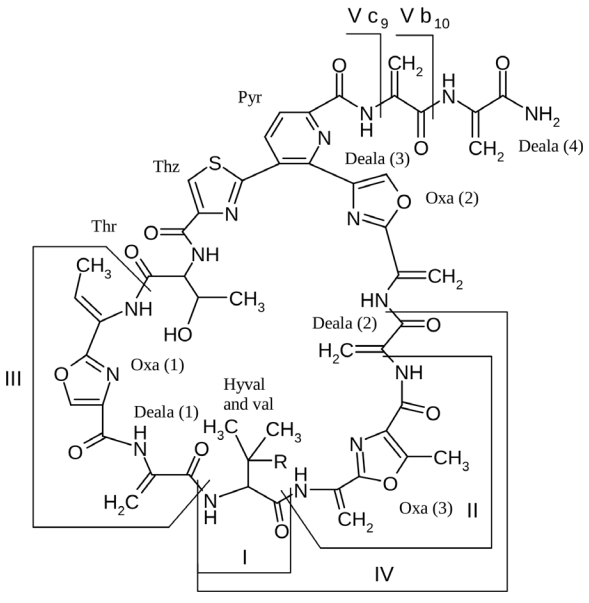

Fig. 2. Selected CID-MS/MS key fragments of the $[M+H]^+$ precursor ions of geninthiocin (**1**, R = OH) and Val-geninthiocin (**2**, R = H) obtained on a quadrupole ion trap mass spectrometer; for detailed information, see Table 1.

the conditions described. Also the MS^2 spectra of $[M+Na]^+$ and $[M–H]^-$ provided little sequence information. Therefore, CID-MS/MS studies were concentrated on the more abundant $[M+H]^+$ species (Fig. 1).

Beside an unspecific loss of H_2O, CO, and NH_3, the following main fragmentation pathways were observed (Latin numbers correspond to the fragments indicated in Fig. 2):

I. Cleavage of a single amino acid residue unit from the peptide ring system was found basically for Hyval (−115 Dalton) in **1** and Val (−99 Dalton), respectively, for **2** (cleavage of two CO–NH peptide bonds). Therefore, the structural difference between both compounds could be allocated at this amino acid at a very early stage.

II. Cleavage of a single amino acid residue unit from the peptide ring system by fragmentation of one CO–NH peptide bond and one CH_2=C–NH bond (H-Oxa-NH_2, H-Hyval/Val-NH_2, H-Deala-NH_2) was detected. Obviously, the -NH–C=CH_2- bond between Oxa(1)-Deala(1) and Oxa(3)-Deala(2) as well as between Deala(2)-Oxa(2) and Hyval/Val-Oxa(3) can be broken favorably leading to a second preferred fragmentation pathway beside peptide bond (-CO–NH-) cleavage.

III. Cleavage of Oxa-Deala dipeptide units with high preference resulted in the prominent fragment ion peaks at m/z = 913 (**1**) and 897 (**2**), respectively. Furthermore, fragmentation of Deala-Hyval/Val occurred with lower intensity.

IV. Cleavage of the tripeptide unit Hyval/Val-Oxa-Deala gave the key fragment at m/z = 798. After cleavage of the tripeptide unit Hyval/Val-Oxa-Deala, a further dipeptide Oxa-Deala loss resulted in the fragment ion m/z = 579 (IV+III).

V. Fragmentation of the linear peptide unit predominantly occurred by cleavage of the NH–CO bond between Deala(4) and Deala(3) forming a b-fragment ion (Vb_{10}). Furthermore, formation of a c-type fragment (Vc_9) by cleavage of a CH_2=C–NH bond between Deala(3) and Pyr induced by the dehydroalanine structure was observed.

The fragmentation pathways derived from collision activated dissociation using a quadrupole ion trap (Table 1) were confirmed by high-resolution MS/MS in an FT-ICR mass spectrometer providing the elemental composition of key fragments (Table 2). By comparison of MS^2 spectra, the position of valine in **2** was determined unambiguously. The information obtained on fragmentation pathways can be applied in further investigations on this class of substances to identify small amounts of derivatives.

The ^1H and ^{13}C NMR spectroscopic data of geninthiocin (**1**) and Val-geninthiocin (**2**) are summarized in Table 3. The ^{13}C NMR spectrum of Val-geninthiocin (**2**) revealed the presence of 50 carbon atoms as for geninthiocin (**1**), in accordance with the empirical formula. While the ^1H NMR spectrum of **2** showed 49 protons, of which 12 were D_2O exchangeable, geninthiocin (**1**) exhibited 13 exchangeable protons. This pointed to one hydroxy group less in **2**, in accordance with the empirical formula. The respective hydroxy signal (δ = 5.17) in geninthiocin (**1**) was replaced in **2** by the methine hydrogen signal of an isopropyl unit. Accordingly, the singlets of the geminal dimethyl group of hydroxyvaline in geninthiocin (**1**) at δ = 1.22 and 1.20 were substituted in **2** by a 6H doublet (δ = 0.97, $J \sim 6.6$ Hz), whose coupling partner gave a multiplet at δ = 2.20. An isobutyl fragment was further confirmed by the ^{13}C NMR spectra of **2**, which exhibited the replacement of the oxygenated β-carbon signal (δ = 71.0) of hydroxyvaline in **1** by a methine signal (δ = 29.5) in compound **2**. Besides the values of hydroxyvaline, all other shifts were nearly identical with those of **1**. The NMR data confirmed

Table 3. ^{13}C and ^{1}H NMR data of geninthiocin (**1**) and Val-geninthiocin (**2**) in [D_6]DMSO in comparison with literature data.

Unit	Position	Geninthiocin (**1**) (lit. data) [21]		Geninthiocin (**1**) (exp. data)		Val-Geninthiocin (**2**)	
		δ_C	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)[b]	δ_C[a]	δ_H (J in Hz)[b]
Thiazole	C-2	163.2	–	163.1	–	163.0	–
	C-4	149.4	–	149.4	–	149.4	–
	CH-5	126.9	8.49	126.7	8.49 (s)	126.6	8.49
	CO	159.9	–	159.9	–	159.8	–
Threonine	NH	–	8.00 (d, 9.0)	–	8.00 (d, 9.0)		8.00 (d, 9.2)
	αCH	57.8	4.60 (dd, 9.0, 3.0)	57.7	4.60 (dd, 9.0, 3.0)	57.6	4.60 (dd, 9.0, 3.0)
	βCH	67.3	4.29 (m)	67.2	4.29 (m)	67.3	4.29 (m)
	γCH$_3$	20.5	1.14 (d, 6.2)	20.4	1.14 (d, 6.2)	20.3	1.14 (d, 6.1)
	OH	–	4.98 (d, 5.5)	–	4.98 (d, 5.5)	–	4.95 (d, 5.4)
	CO	168.8	–	168.7	–	168.6	
Oxazole (1)	NH	–	9.60	–	9.55 (s)	–	9.60 (s br)
	αC	123.1	–	123.1	–	123.0	–
	βCH	129.5	6.55 (q, 7.3)	129.5	6.55 (q, 7.3)	129.2	6.53 (q, 7.0)
	γCH$_3$	13.8	1.74 (d, 7.3)	13.7	1.74 (d, 7.3)	13.6	1.75 (d, 7.0)
	C-2	159.4	–	159.4	–	159.4	–
	C-4	136.1	–	136.0	–	136.1	–
	CH-5	142.7	8.70	142.6	8.70 (s)	142.5	8.70 (s)
	CO	158.4	–	158.3	–	158.3	–
Dehydroalanine (1)	NH	–	9.39	–	9.39 (s)	–	9.40 (s br)
	αC	133.4	–	133.4	–	133.3	–
	βCH$_2$	103.7	6.46, 5.88	103.7	6.46, 5.88	105.8	6.46 (s), 5.88 (s)
	CO	163.7	–	163.7	–	163.7	–
Hydrovaline and Valine	NH	–	8.23 (d, 8.5)		8.23 (d, 8.5)		8.25 (d, 8.1)
	αCH	61.8	4.64 (d, 8.5)	61.7	4.64 (d, 8.5)	60.0	4.61 (dd, 8.8, 2.8)
	βC	71.0	–	71.0	–	29.5	2.20 (m)
	γCH$_3$	27.3	1.22	27.3	1.22 (s)	18.9	0.97 (d, 6.6)
	γCH$_3$	26.1	1.20	26.0	1.20 (s)	18.8	0.97 (d, 6.6)
	OH	–	5.17	–	5.17 (s)	–	–
	CO	169.4	–	169.3	–	171.0	–
Oxazole (3)	NH	–	9.61	–	9.60	–	9.60
	αC	128.5	–	128.5	–	128.6	–
	βCH$_2$	105.6	6.13, 5.65	105.7	6.13, 5.65	106.1	6.09, 5.70
	C-2	155.2	–	155.1	–	155.1	–
	C-4	129.2	–	129.2	–	129.2	–
	C-5	154.6	–	154.5	–	154.2	–
	CH$_3$-5	11.5	2.62	11.5	2.62	11.4	2.63 (s)
	CO	159.5	–	159.5	–	159.4	–
Dehydroalanine (2)	NH	–	9.36	–	9.36	–	9.37 (s br)
	αC	133.9		133.9		133.9	
	βCH$_2$	105.9	6.36, 5.76	105.9	6.36, 5.76	105.8	6.36, 5.76
	CO	162.7		162.7		162.6	
Oxazole (2)	NH	–	9.77	–	9.77	–	9.69
	αC	129.3		129.1		130.0	
	βCH$_2$	111.2	5.70, 5.71	111.0	5.70, 5.71	111.2*	5.70, 5.73
	C-2	158.1	–	158.3	–	158.0	–
	C-4	139.2	–	139.2	–	139.1	–
	CH-5	140.5	8.68	140.5	8.68	140.4	8.68 (s)
Pyridine	C-2	149.2	–	149.2	–	149.2	–
	C-3	130.2	–	130.1	–	130.1	–
	CH-4	141.0	8.50 (d, 8.0)	141.0	8.50 (d, 8.0)	140.8	8.52 (d, 8.2)
	CH-5	121.5	8.23 (d, 8.0)	121.3	8.23 (d, 8.0)	121.4	8.25 (d, 8.1)
	C-6	146.8	–	146.8	–	146.8	–
	CO	161.6	–	161.6	–	161.5	–
Dehydroalanine (3)	NH	–	10.53	–	10.53	–	10.51 (s br)
	αC	134.7	–	134.7	–	134.7	–
	βCH$_2$	106.2	6.42, 5.83	106.1	6.42, 5.83	106.0*	6.43, 5.84
	CO	162.0	–	162.0		162.0	
Dehydroalanine (4)	NH	–	9.44	–	9.44	–	9.42 (s br)
	αC	135.1	–	135.0	–	135.0	–
	βCH$_2$	106.0	6.03, 5.69	106.0	6.03, 5.69	106.0	6.05, 5.70
	CO	165.1	–	165.1	–	165.0	–
	NH$_2$	–	7.93, 7.50	–	7.93, 7.50	–	7.92, 7.48

[a] (75 MHz); [b] (300 MHz); * expected value.

the MS-derived structure **2**, for which we suggest the name Val-geninthiocin. According to the NMR and OR data, our **1**-sample had the same absolute configuration as reported for geninthiocin in the literature [21]; the same configuration is plausible also for **2**.

Biological activities

Biological activities of geninthiocin (**1**), Val-geninthiocin (**2**) and chalcomycin were measured using the agar diffusion method. In comparison with **1**, compound **2** showed slightly lower antibacterial activities against Gram-positive bacteria, *viz. Bacillus subtilis*, *Staphylococcus aureus* and *Streptomyces viridochromogenes* (Tü 57), and minor antifungal activity against *Mucor miehei* (Tü 284) and *Candida albicans*. Both compounds exhibited, however, no activities against the Gram-negative *Escherichia coli* and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* (Table 4).

Table 4. Antibacterial and antifungal activities of geninthiocin (**1**) in comparison with Val-geninthiocin (**2**) and chalcomycin (conc. 40 μg per disk).

Tested microorganisms	Inhibition zone ∅ (mm)		
	Genin-thiocin (**1**)	Val-Genin-thiocin (**2**)	Chalco-mycin
Bacillus subtilis	15	13	29
Staphylococcus aureus	15	14	36
Streptomyces viridochromogenes (Tü 57)	14	11	37
Escherichia coli	–	–	–
Candida albicans	11	11	11
Mucor miehei (Tü 284)	11	11	11
Chlorella vulgaris	–	–	–
Chlorella sorokiniana	–	–	–
Scenedesmus subspicatus	–	–	–

Experimental Section

Optical rotations: Polarimeter (Perkin-Elmer, model 243). UV/Vis spectra were recorded on a Perkin-Elmer Lambda 15 UV/Vis spectrometer. NMR spectra were measured on Varian Unity 300 and Varian Inova 600 spectrometers. Electrospray ionization mass spectrometry (ESI-MS): Finnigan LCQ ion trap mass spectrometer. High-resolution mass spectra (HRMS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). R_f values were measured on a Polygram SIL F/UV$_{254}$ system (Merck, pre-coated sheets).

MS/MS studies

Samples of geninthiocin (**1**) and Val-geninthiocin (**2**) were dissolved in methanol/water (75/25) containing 0.1 % formic acid. Low resolution MS^2 and MS^3 measurements were performed on an LCQ quadrupole ion trap instrument (Finnigan, San Jose, USA) using electrospray ionization in the positive and negative ionization mode with an electrospray voltage of $+/-$ 4.5 kV. Samples were introduced by means of a syringe pump with a flow rate of 3 μL/min. The capillary was heated to 200 °C. $[M+H]^+$, $[M+2H]^{2+}$, $[M+Na]^+$, and $[M–H]^-$ ions were isolated (isolation width 3.0 Dalton) and submitted to fragmentation by collision-induced dissociation with helium as collision gas. For MS^3 investigations fragment ions were isolated (isolation width 3.0 Dalton peak width) and fragmented under the same conditions.

Accompanying high-resolution MS/MS investigations were performed on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer. Samples were infused with a flow rate of 2 μL/min. CID-MS/MS studies were carried out on the $[M+H]^+$ species inside the ICR cell with argon as collision gas. Conditions: electrospray voltage 4.2 kV, capillary exit voltage 100 V, nebulizing gas nitrogen (30 psi), drying gas nitrogen (250 °C), hexapole accumulation 0.1 s, mass range m/z = 100 – 1400.

Taxonomy of the producing strain

The *Streptomyces* sp. RSF18 was isolated from the soil of a rose field at the province Punjab, Pakistan. The taxonomic status of the strain RSF18 was determined by preliminary physiological testing and 16S rRNA gene sequencing. Cultural characteristics were observed during the incubation at 27 °C for 21 d on GYM medium [27]. It formed a pale yellowish substrate mycelium, which changed to dark brown after 14 d of incubation. The aerial mycelium was powdery and characteristically whitish to grey in color on prolonged incubation. Melanoid pigment was produced, and a soluble, slightly orange pigment was formed. For the cultural characteristics, physiological properties, and utilization of carbon sources of strain RSF18 see Table 5. Permissive temperature ranges for growth of the strain RSF18 were 20 to 37 °C with an optimum at 28 °C. Comparison of these characteristics with those of actinomycete species described in Bergey's Manual of Systematic Bacteriology [28] strongly suggested that strain RSF18 belongs to the genus *Streptomyces*. Taxonomic determination, PCR amplification and 16S rRNA gene sequencing were performed as described previously [29]. The nucleotide sequence of 1418 bp (accession number EU294139) of the 16S rRNA gene of the *Streptomyces* sp. RSF18 was determined in both strands. The alignment of this sequence through matching with reported 16S rRNA gene sequences in the gene bank showed high similarity (98 – 99 %) to *Streptomyces* 16S rRNA genes.

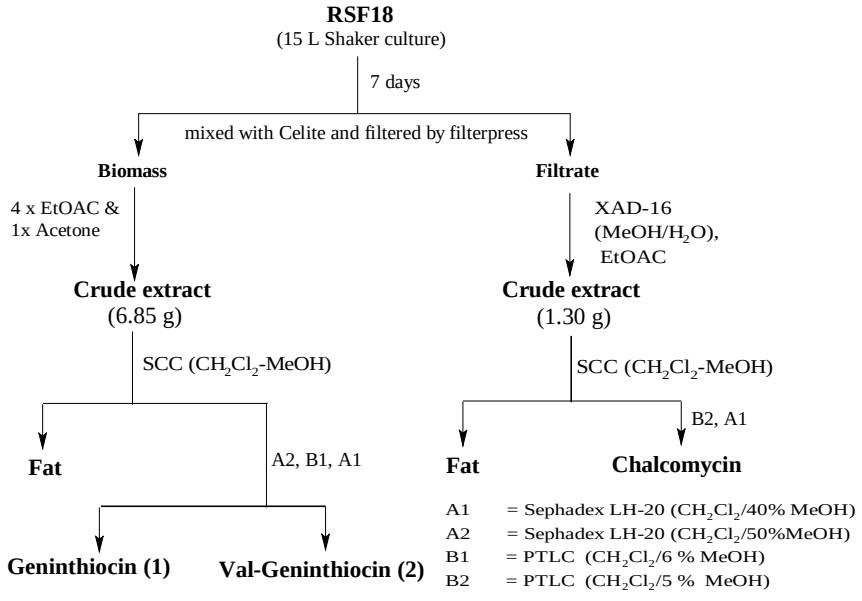

Fig. 3. Work-up scheme of the terrestrial *Streptomyces* sp. RSF18.

Table 5. Cultural and physiological characteristics of strain RSF18[a].

Growth pattern	well grown ~ partitioned
Substrate mycelium	yellowish ~ dark brown
Ariel mycelium	whitish ~ grey
Growth temperature range	20 ~ 37 °C
Production of melanin	+
Utilization of organic acids	+
Utilization of oxalates	+
Hydrolysis of urea	+
Hemolysis	−
Carbon source utilization	
D-Glucose	+
L-Arabinose	+
Sucrose	−
D-Fructose	+
D-Mannitol	+
Raffinose	+
D-Galactose	−
Soluble starch	+
Glycerol	+

[a] +: Positive; −: negative.

M_2 medium

A solution of 10 g malt extract, 5 g yeast extract and 5 g glucose in 1 L of tap water was set to pH = 7.8 with 2 N NaOH and sterilized for 30 min at 121 °C.

Fermentation of isolate RSF18

The terrestrial isolate *Streptomyces* sp. RSF18 was inoculated from its storage culture on three M_2 agar plates and incubated for 96 h at 28 °C. The well-developed colonies were used to inoculate 60 of 1 L Erlenmeyer flasks each containing 250 mL of M_2 medium, and further incubated for 7 d as shaker culture (95 rpm) at 28 °C. The resulting yellowish brown culture broth was mixed with *ca.* 1 kg diatomaceous earth, pressed through a pressure filter giving filtrate and biomass, which were extracted separately. The filtrate was subjected to adsorption on Amberlite XAD-16, and the resin subsequently extracted with methanol. The methanol extract was concentrated, and the resulting aqueous residue was re-extracted with ethyl acetate followed by evaporation *in vacuo* to dryness, affording 1.30 g of brown extract. The mycelia phase was extracted 3 times with ethyl acetate followed by acetone (1 time). The acetone was evaporated from the aqueous residue, which was finally extracted with ethyl acetate. The combined ethyl acetate extracts were concentrated *in vacuo* to dryness giving 6.85 g of brown extract.

Isolation

Fractionation and purification of the mycelia extract (6.85 g) using silica gel column chromatography and elution with a CH_2Cl_2/MeOH gradient gave two crude fractions of **1** and **2**. Further purification by PTLC and Sephadex LH-20 resulted in two colorless solids of geninthiocin (**1**; 78.6 mg) and Val-geninthiocin (**2**; 27.5 mg). Chromatography of the filtrate extract (1.3 g) using silica gel with a CH_2Cl_2-MeOH gradient followed by PTLC and Sephadex LH-20 (see Fig. 3) delivered chalcomycin as a white solid (19.8 mg).

*Geninthiocin (**1**)*

White amorphous solid. Yellow with anisaldehyde/sulfuric acid spraying reagent. Soluble in DMSO, MeOH,

EtOH and EtOAc. Insoluble in hexane and CH_2Cl_2. – R_f = 0.55 (CH_2Cl_2/10 % MeOH). – ^{1}H NMR ([D$_6$]DMSO, 300 MHz) and ^{13}C NMR ([D$_6$]DMSO, 75 MHz): see Table 3. – $[\alpha]_D^{25}$ = +155 (*c* = 0.1, MeOH). – UV/Vis (MeOH): λ_{max}(lg ε_{max}) = 237 nm (5.09); (MeOH/HCl): 238 nm (5.04); (MeOH/NaOH): 238 nm (5.04). – (+)-ESIMS: *m*/*z* = 1154.3 $[M+Na]^+$. – (−)-ESIMS: *m*/*z* = 1130.2 $[M-H]^-$. – (+)-HRESIMS: *m*/*z* = 1132.33319 (calcd. 1132.33260 for $C_{50}H_{50}N_{15}O_{15}S$, $[M+H]^+$), 1154.31444 (calcd. 1154.31455 for $C_{50}H_{49}N_{15}O_{15}SNa$, $[M+Na]^+$).

*Val-Geninthiocin (**2**)*

White amorphous solid. Yellow with anisaldehyde/sulfuric acid spraying reagent. Soluble in DMSO, MeOH, EtOH and EtOAc. Insoluble in hexane and CH_2Cl_2. – R_f = 0.61 (CH_2Cl_2/10 % MeOH). – ^{1}H NMR ([D$_6$]DMSO, 300 MHz) and ^{13}C NMR ([D$_6$]DMSO, 75 MHz): see Table 3. – $[\alpha]_D^{25}$ = +131 (*c* = 0.1, MeOH); UV (MeOH): λ_{max}(lg ε_{max}) = 237 nm (4.93); (MeOH/HCl): 239 (4.92), 205 nm (4.94); (MeOH/NaOH): 239 nm (4.92). – (+)-ESIMS: *m*/*z* = 1138.4 $[M+Na]^+$. – (+)-HRESIMS: *m*/*z* = 1116.33707 (calcd. 1116.33769 for $C_{50}H_{50}N_{15}O_{14}S$, $[M+H]^+$), 1138.31812 (calcd. 1138.31963 for $C_{50}H_{49}N_{15}O_{14}SNa$, $[M+Na]^+$).

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