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Microbial Products. II.[1] Granaticinic Acid, a New Antibiotic From a Thermophilic Streptomycete

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Streptomyces sp. XT-11989 produces a mixture of two antibiotics with litmus-like indicator properties. One of them was shown to be identical with granaticin, the other was identified as [1S,3S,4S,7R,9R,10S,13R]-4,7,9,10-tetrahydro-5,12-dioxo-4,6,10,11,13-pentahydroxy-1,9-dimethyl-(1*H*,3*H*)-7,10-ethanonaphtho[2,3-c: 6,7-c']dipyran-3-acetic acid and termed granaticinic acid. Microbial production and nuclear magnetic resonance data of these antibiotics are discussed and the antibacterial properties of the antibiotics are compared.

(Keywords: Antibiotic; Granaticinic Acid; NMR spectra)

Mikrobielle Produkte. II. Granaticinsäure, ein neues Antibiotikum von einem thermophilen Streptomyceten

Der Streptomycetenstamm XT-11989 produziert eine Mischung von zwei Antibiotika mit Lakmus-ähnlichen Indikatoreigenschaften. Eines dieser Antibiotika erwies sich als Granaticin, das andere wurde als [1S,3S,4S,7R,9R,10S,13R]-4,7,9,10-tetrahydro-5,12-dioxo-4,6,10,11,13-pentahydroxy-1,9-dimethyl-(1*H*,3*H*)-7,10-ethanonaphto[2,3-c: 6,7-c']dipyran-3-essigsäure identifiziert und Granaticinsäure benannt. Mikrobiologische Produktion und Kernresonanzdaten dieser Antibiotika werden besprochen und ihre antibakteriellen Eigenschaften verglichen.

Introduction

Pigments with litmus-like indicator properties are quite commonly produced by actinomycetes[2]. The chemical structure of granaticin (**1**), a typical representative of this group of microbial products, is known in detail[3,4]. It is primarily active against Gram-positive microorganisms[2] and shows significant antitumor activity against P-388 lymphocytic

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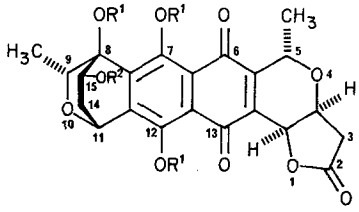
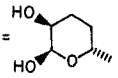
leukemia in mice and cytotoxicity against KB cells[5]. Identical with antibiotic WR 141[6a] and litmomycin[5], **1** is a metabolite of a number of actinomycetes, notably streptomycetes[2,5,6,7,8,9], and occurs either as the sole antibiotic component[8,5] or together with a second one[8,9], identified as granaticin L-rhodinoside and termed granaticin B (**2**)[8,3,4].



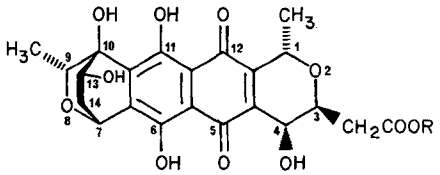
1: $R^1 = R^2 = H$ (Granaticin)



2: $R^1 = H$, R^2 = (Granaticin B)

3: $R^1 = R^2 = CH_3CO$ (Tetra-O-acetylgranaticin)

In addition to these antibiotics, some granaticin-producers have been reported to elaborate minor pigments[2] devoid of antibacterial activity[6a]. Recently, 4-deoxygranaticinic acid(4-deoxy-**4**) has been described[6b] and found to be a precursor in the biosynthesis of granaticin[6c].



4: R = H (Granaticinic acid)

5: $R = CH_3$ (Granaticinic acid methyl ester)

Results and Discussion

We have recently isolated a mixture of two antibiotics from the culture of a thermophilic streptomycete sp. XT-11989. The major component proved to be identical with granaticin (**1**); the minor one exhibited generally weaker antibiotic properties than **1** (Table 3) and

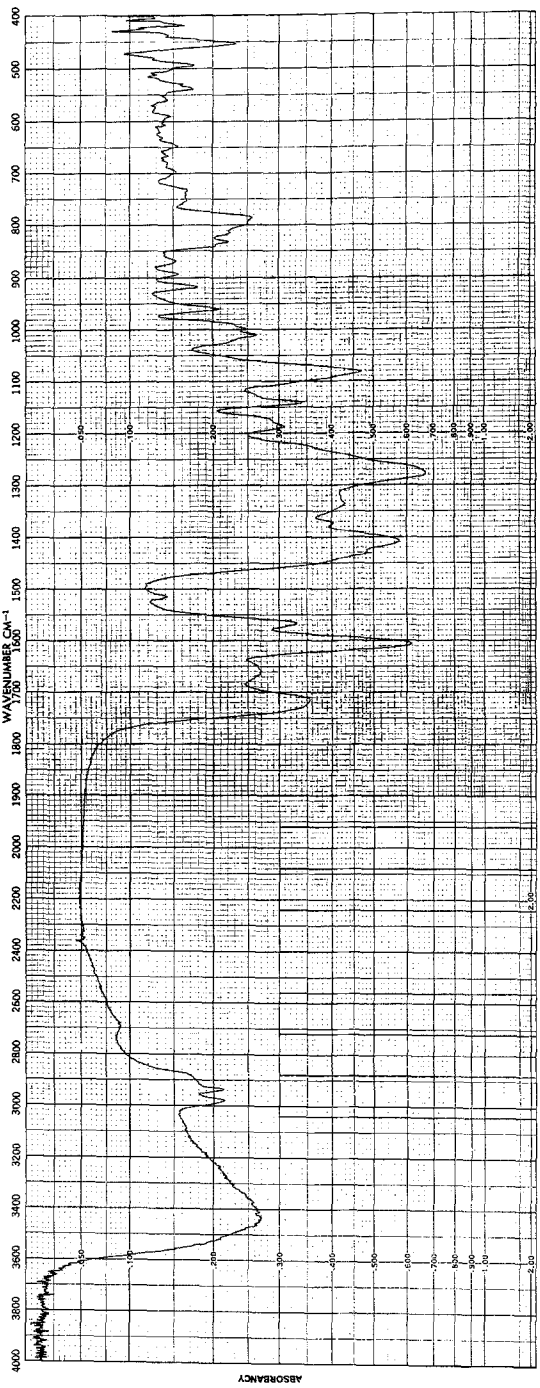


Fig. 1. IR spectrum of granaticinic acid in KBr

was shown to be [1S,3S,4S,7R,9R,10S,13R]-4,7,9,10-tetrahydro-5,12-dioxo-4,6,10,11,13-pentahydroxy-1,9-dimethyl-(1*H*,3*H*)-7,10-ethano-naphtho-[2,3-c:6,7-c']dipyran-3-acetic acid (**4**), appropriately termed granaticinic acid, in view of its similarity to granaticin.

The structure **4** is supported by the following evidence. The IR spectrum of **4** (Fig. 1) shows typical OH stretching vibrations of the carboxylic acid at $3000 \sim 2650\,cm^{-1}$ and a band at $1715\,cm^{-1}$ indicative of carbonyl vibrations of a saturated aliphatic carboxylic acid which disappear upon conversion of the acid to the triethylamine salt. Diazomethane treatment converted acid **4** to methyl ester **5** with an ester-carbonyl stretching absorption at $1735\,cm^{-1}$ as opposed to $1785\,cm^{-1}$ in **1**. Under acidic conditions, such as trifluoroacetic acid at room temperature, **4** was rapidly lactonized to **1** and upon further treatment with acetic anhydride and sulfuric acid a crystalline tetra-O-acetyl derivative was formed exhibiting the same elemental composition, spectral properties and optical rotation as reported for **3**[2]. The absolute configuration of **4** is, therefore, identical with that of granaticin. ^{13}C- and ^1H-NMR spectral data of **1**, **4** and **5** are compared in Tables 1 and 2, respectively. To permit direct comparisons, the numbering system of granaticinic acid and its ester was also employed for granaticin.

The ^{13}C-NMR spectrum of granaticinic acid was assigned on the basis of empirical chemical-shift correlations, single frequency off-resonance (SFOR) residual spin-spin coupling constants and spectral comparison with **1** and **5**.

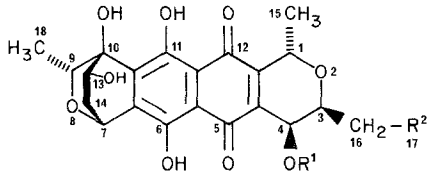
The methyl groups 18 and 15 of granaticinic acid (**4**) exhibited ^1H doublets ($J = 6.5\,Hz$) at $\delta\,0.96$ and 1.54, respectively, confirmed by proton-proton decoupling from methine quartets at $\delta\,3.73$ and 5.05, respectively, and showed ^{13}C quartets in the SFOR spectra at δ 16.8 and 17.7 respectively.

The methylene carbon 16 was assigned to the signal at $\delta\,35.9$, as it shifted paramagnetically by some 1 ppm upon lactonization to **1**. The other methylene group of **4** at $\delta\,36.7$ shifted diamagnetically, but only by 0.2 ppm, and was therefore assigned to C-14, the methylene group remote from the lactonization site.

Since their chemical shifts in 6:1 $CDCl_3$/*DMSO*-d_6 solution were not affected (within $\pm$ 0.01 ppm) in the transformation from granaticinic acid to **1**, the methine carbons 7, 9 and 13 were distinguished from 1, 3 and 4 which were affected. Carbons within each group were differentiated from each other by their residual SFOR coupling constants. The methine band at $\delta\,58.7$ shifted dramatically (+ 10.0 ppm, downfield) in this transformation and was thus confirmed as 4.

The highest-field singlet at $\delta\,80.5$ in the SFOR spectrum was assigned to C-10, the only sp^3-hybridized quaternary carbon in the molecule.

Table 1. ^{13}C-NMR *spectral data of granaticinic acid and derivatives*

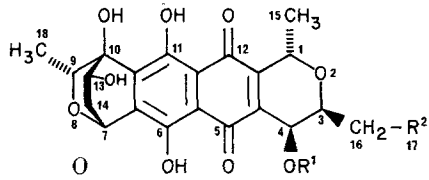


Carbon	**1**: $R^1 + R^2 = -\overset{\overset{O}{\|}}{C}-$ (Granaticin) **a**	**b**	**4**: R^1 = H, R^2 = COOH (Granaticinic acid) **b**	**5**: R^1 = H, R^2 = $COOCH_3$ (Granaticinic acid methyl ester) **a**
15	18.1	18.1	17.7	17.6
18	16.6	16.8	16.8	16.5
16	36.9	36.9	35.9	35.6
14	35.6	36.5	36.7	35.6
3	66.8	66.6	67.7	67.3
4	68.9	68.7	58.7	59.9
13	70.9	70.9	70.7	70.8
1	66.1	66.1	67.1	67.8
9	72.6	72.3	72.1	72.6
7	61.7	61.6	61.6	61.8
10	80.5	80.3	80.2	80.5
11a	110.2	110.1	110.2	110.2
5a	111.4	111.4	110.8	110.8
12a	130.5	130.4	137.7	137.1
10a	138.2	139.6	139.8	138.9
6a	145.0	144.3	141.9	143.5
4a	145.1	145.1	144.8	144.2
12	164.9	165.5	161.9	166.2
11	166.5	167.2	167.9	171.3
6	171.3	170.4	172.9	171.5
5	178.0	176.8	175.5	171.8
17	174.2	174.2	175.5	172.2
OCH_3				51.8

a: $CDCl_3$ as solvent, **b**: $CDCl_3$ — *DMSO-d_6*, 6:1, *v/v*, as solvent.

The ring-junction carbons C-11a and C-5a were assigned according to the literature values for naphtharizin[10]. They were differentiated by the shift of only one of these carbons in the conversion from **1** to **5**. The carbon which shifted was assigned to C-5a, the carbon closer to the lactonization center. Four other sp^2-hybridized carbons at ring junctions (4a, 6a, 10a and 12a) were grouped together at lower field with-

Table 2. ^{1}H-NMR *spectral data of granaticinic acid and derivatives*



1: $R^1 + R^2 = -\overset{\overset{O}{\|}}{C}-$ **4**: $R^1 = H$, $R^2 = COOH$ **5**: $R^1 = H$, $R^2 = COOCH_3$

Carbon	(Granaticin) **a**	(Granaticinic acid) **b**	(Granaticinic acid methyl ester) **a**
16	2.67, 2.98 (ABX...) $J_{gem} = 18$ Hz $J_{vic} = 0, 4.5$ Hz	2.73, 2.73 (d) $J_{vic} = 6.5$ Hz	2.84, 2.84 (d) $J_{vic} = 6.5$ Hz
3	4.73 (dd) $J_{3,16} = 4.5$ Hz $J_{3,4} = 3.5$ Hz	4.28 (t) $J_{3,16} = 6.5$ Hz	4.38 (dt) $J_{3,16} = 6.5$ Hz $J_{3,4} = 2.5$ Hz
1	5.23 (q) $J_{1,15} = 6.5$ Hz	5,03 (q) $J_{1,15} = 6.5$ Hz	5.09 (q) $J_{1,15} = 6.5$ Hz
15	1.58 (d) $J_{1,15} = 6.5$ Hz	1.54 (d) $J_{1,15} = 6.5$ Hz	1.59 (d) $J_{1,15} = 6.5$ Hz
9	3.80 (q) $J_{9,18} = 6.5$ Hz	3,74 (q) $J_{9,18} = 6.5$ Hz	3.77 (q) $J_{9,18} = 6.5$ Hz
18	1.02 (d) $J_{9,18} = 6.5$ Hz	0.97 (d) $J_{9,18} = 6.5$ Hz	1.01 (d) $J_{9,18} = 6.5$ Hz
7	5.19 (broad s)	5.13 (broad s)	5.17 (dd) $J_{7,14\alpha} = 3$ Hz $J_{7,14\beta} = 2$ Hz
4	5.32 (d) $J_{3,4} = 3.5$ Hz	4.66 (broad s)	4.72 (d) $J_{3,4} = 2.5$ Hz
14[a]	1.58 (dt), 2.78 (ddd) $J_{gem} = 15$ Hz $J_{7,14\beta} = J_{13,14\beta} = 2$ Hz $J_{7,14\alpha} = 3, J_{13,14\alpha} = 8$ Hz	1.47, 2.65	1.51, 2.71
13	4.01 (dd) $J_{13,14\alpha} = 8$ Hz $J_{13,14\beta} = 2$ Hz	3.94 (d, broad) $J_{13,14\alpha} = 8$ Hz $J_{13,14\beta} \sim 2$ Hz	4.00 (dd) $J_{13,14\alpha} = 8$ Hz $J_{13,14\beta} = 2$ Hz

a: $CDCl_3$ as solvent, **b**: $CDCl_3$—*DMSO-d_6*, 6:1, *v/v*, as solvent.

[a] The α-designation refers to the proton on C-14 closer to the ring oxygen.

Tabelle 3. *Antimicrobial properties*[a] *of granaticin* (**1**) *and granaticinic acid* (**4**)

Test organism	Diameter of inhibition zones in mm, at antibiotic concentrations in μg/ml					Ratio of Activity, **1/4**
	1			**4**		
	500 μg	50 μg	3.1 μg	500 μg	50 μg	
Staphylococcus aureus, ATCC 6538P	29.5	23.8	15.2	22.5	15.5	14
Sarcina lutea, ATCC 9341	29	20.2	12	18	trace	10
Bacillus megatherium, ATCC 8011	—	32.2	21.3	—	21.1	16
Bacillus sp. E, ATCC 27859	35.3	27.5	trace	21.3	15.5	20
Bacillus subtilis, NRRL 558	—	27.4	20.2	—	20.5	16
Bacillus sp. TA, ATCC 27860	—	26.5	19.4	—	19.7	16
Streptomyces cellulosae, ATCC 3313	34.5	25.8	11.3	25.5	12.6	12
Acinetobacter calcoaceticus, ATCC 10153	19	11.8	0	11.6	0	10
Serratia sp. 101, ATCC 93	15.6	0	0	12.8	0	5

[a] Both compounds were tested at 500, 250, 100, 50, 25, 12.5, 6.25, 3.13 and 1.57 μg/ml; representative values are presented in the table. Ratios of activity were estimated from the concentrations required to produce equivalent zone sizes.

in a 7.1 ppm range, whereas the four oxygenated sp^2-carbons (5, 6, 11 and 12) are grouped in a 13.6 ppm range. The lactone carbonyl exhibited no solvent shift. The carboxyl groups of **4** and **5** were assigned on the basis of the absence in the acid, and presence in the ester, of SFOR residual coupling constants.

The ^{1}H-NMR spectrum of granaticin in $CDCl_3$ including proton-proton decoupling experiments was assigned largely on the basis of the literature cited, but allowing for solvent shifts. Spectra of granaticinic acid and its methyl ester were interpreted by comparison with those of granaticin. In particular, H-4 at δ 5.32 was shifted diamagnetically to 4.66 in granaticinic acid, whereas the protons at C-16 exhibited magnetic equivalence.

Both **1** and **4** exhibited good antibiotic properties against Gram-positive bacteria, especially cocci and rods (Table 3), but were only weakly active against two Gram-negative bacteria, and inactive against the yeasts *Saccharomyces cerevisiae* ATCC 4226, and *Candida albicans* NRRL 477, and the fungi *Paecilomyces varioti* ATCC 26820 and *Penicillium digitatum*, ATCC 26821. Although the inhibition zones themselves were qualitatively similar, **1** was 5 to 20 times as active as **2**.

Experimental

The microorganism was cultivated in media A (Difco Thermoactinomyces fermentation medium[11]), B (1% yellow split pea meal, 1% cornstarch, 0.1% calcium carbonate and 0.1% dipotassium hydrogen phosphate, pH = 7.5 after sterilization), and C (0.5% dried tomato pomace, 0.5% distiller's dried solubles, 0.5% meat peptone paste, 0.5% dried debittered yeast, 2% cornstarch, 0.1% calcium carbonate and 0.1% dipotassium hydrogen phosphate, pH = 7.0 after sterilization).

Antimicrobial activities were determined by an agar-well diffusion assay. Antibiotic solutions were placed in 8-mm diameter wells and incubated overnight at 35 °C.

Thin-layer chromatography was performed with precoated silica gel plates (E. Merck, Darmstadt) and systems A (chloroform — methanol, 4:1, *v/v*) and B (chloroform — methanol, 8:1, *v/v*).

IR spectra were recorded on a Digilab FT spectrophotometer, Modell 14, in KBr discs.

NMR spectra were obtained with a Varian XL-100 spectrometer operating in CW mode for ^{1}H and FT mode for ^{13}C. With *DMSO-d_6* as solvent, all ^{1}H-NMR signals of **1** and **4** were rather broad and no useful ^{13}C-NMR spectra could be obtained. Addition of $CDCl_3$ to solutions in *DMSO-d_6* reduced line broadening significantly.

Fermentations

Streptomyces sp. XT-11989* was isolated from a Japanese soil sample and grew well on agar plates and in liquid media in shaken flasks at 28°, 35°, 48° and

* This culture was not further identified taxonomically, since a thermophilic streptomycete has already been reported to be a producer of granaticin[7].

55°. Media A, B and C were dispensed in 75-ml portions into 500-ml baffled Erlenmeyer flasks and each inoculated with 0.75 ml of a culture in the same medium previously developed under submerged conditions at 48° for 15 1/2 h.

Incubation for 22 to 27 h at 48° or 55° on a rotary shaker at 250 rpm gave better microbial growth in medium B than in A, although good antibiotic activities were observed in both media. Fermentation in medium C at 48° for 22 h, however, yielded antibiotic activities which were some ten times higher than those attainable in medium A. Coincident with the development of antibiotic activity was the increase in color from pale red to grape-purple to blue-black.

A stainless steel tank containing medium C (240 liters) and SAG-431 antifoam (0.24 liters) was inoculated with a vegetative liquid culture (2 liters) previously incubated for 8 h at 48°. The tank contents were stirred at 280 rpm, aerated at a rate of 0.085 m^3/min and maintained at 48°. As the purple color developed somewhat slowly, the fermentation time was extended to 40 h. The whole broth was adjusted to pH 2.5 with dilute hydrochloric acid, mixed with Hyflo Supercel and centrifuged. The clear solution was extracted with chloroform (2 × 114 liters) and the pooled extracts were washed with water (50 liters) and concentrated under reduced pressure to a volume of 2.5 liters.

Isolation of granaticin (**1**) *and granaticinic acid* (**4**)

The concentrated chloroform extract was distributed in three separatory funnels by the method of complete withdrawal[12] with a mixture of acetonitrile—hexane employing 500 ml portions of upper and lower phases. The first two lower phases were combined and concentrated to give a dark-red syrup (10 g) which was chromatographed on a column of Sephadex LH-20 (5.5 cm × 24 cm) with acetone as mobile phase eluting **1** (1.24 g) first, followed by **4** (200 mg). Rechromatography of **1** on the same column with methanol and subsequently on a column of Amberlite XAD-2 (200-400 mesh, 2.5 cm × 35 cm) with methanol afforded pure **1**, R_f 0.60 (A) and 0.34 (B), obtained in crystalline form from acetone (0.3 g). Granaticinic acid was further purified by rechromatography on a column of Sephadex LH-20 (2.5 × 45 cm) with methanol as mobile phase yielding a homogeneous red precipitate from acetone and hexane (0.1 g), R_f 0.10 (A) and 0.05 (B).

Granaticinic acid methyl ester (**5**)

An excess of diazomethane in ca. 1 ml of tetrahydrofuran was added to a solution of **4** (100 mg) in acetone (2 ml) at 0°. The stoppered flask was kept at room temperature for 24 hr, the content concentrated and chromatographed on a column of Sephadex LH-20 (2.5 × 45 cm) with acetone as mobile phase to remove unreacted **1** and furnish pure **4** (80 mg), R_f 0.65 (A) and 0.40 (B).

Acknowledgments

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