Parimycin: Isolation and Structure Elucidation of a Novel Cytotoxic 2,3-Dihydroquinizarin Analogue of γ-Indomycinone from a Marine *Streptomycete* Isolate†

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In our screening of actinomycetes from the marine environment for bioactive components, a new antibiotic with a novel structure designated as parimycin (**2**) was obtained from the culture broth of *Streptomyces* sp. isolate B8652. The structure of the new antibiotic was determined by spectroscopic methods and by comparison of the NMR data with those of the structurally related γ-indomycinone (**1a**).

In the course of our screening program for novel bio-active compounds from marine actinomycetes, the ethyl acetate extract of *Streptomyces* sp. isolate B8652 drew our attention due to a striking non polar yellow band on tlc, which gave an orange to violet colouration turning to pink after a few minutes with anisaldehyde/sulphuric acid. The violet colour reaction on treatment with dilute sodium hydroxide solution was typical for *peri*-hydroxy quinones which, however, never show a strong green fluorescence as it was observed here. In addition, high biological activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Streptomyces viridochromogenes* (Tü 57) deserved further interest. The working up of the extract resulted in the isolation of a novel 2,3-dihydro-1,4-anthraquinone which we named parimycin (**2**). In this paper we report the taxonomy of the producing strain, the structure elucidation of **2** as well as the biological activity of this compound.

Taxonomy of the producing strain

The strain B8652 has been derived from a sediment of the Laguna de Terminos at the Gulf of Mexico and was isolated on Oil No.2 agar[1)] containing 50 % natural seawater. The 16S rRNA sequence of the strain B8652 is 99 % similar to that of *Streptomyces chartreusis* (strain ISP 5085) belonging to the *Streptomyces cyaneus* species-group. The substrate mycelium is brown and the aerial mycelium is greenish grey with spiral spore chains (*Spirales*). The surface of the spores is spiny. Melanin pigment is neither produced on peptone-yeast extract-iron agar[2)] nor on tyrosine agar[2)]. Optimum growth temperature is at about 30 °C. The strain does not develop at 10 °C and at 45 °C. Salinity tolerance is low. Growth is only obtained in media with seawater salinity from 0 % up to 3 %. Starch, casein, gelatin, and esculin are degraded. Chitin and cellulose are not hydrolized. The strain is catalase and nitrate reductase positive. H_2S is not produced.

The use of carbon sources was tested with SFN2-Biolog (Hayward, CA, USA) using BMS-N without agar as basal medium[3)] A wide range of organic compounds can be utilized for growth: alaninamide, L-alanine, L-alanyl glycine, γ-amino butyric acid, L-arabinose, D-arabitol, L-asparagine, L-aspartic acid, bromosuccinic acid, cellobiose, citric acid, α-cyclodextrin, dextrin, D-fructose, D-galactose, D-gluconic acid, D-glucosaminic acid, D-glucose, L-glutamic acid, glycerol, glycogen, β-hydroxy butyric acid, α-ketoglutaric acid, D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-mellobiose, methylpyruvate, N-acetyl-

D-glucosamine, propionic acid, putrescine, quinic acid, D-raffinose, L-rhamnose, D-saccharic acid, succinic acid, sucrose, L-threonine, D-trehalose, turanose, tween 40, tween 80.
The reference culture of Streptomyces sp. isolate B8652 is kept on yeast extract-malt extract agar[4)] in the Collection of Marine Actinomycetes at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven.

Fermentation and Isolation

Well-grown agar subcultures of *Streptomyces* sp. isolate B8652 served to inoculate 25 1 l Erlenmeyer flasks each containing 200 ml of yeast extract-malt extract medium[4]. The flasks were incubated with 95 rpm at 28 °C for 3 days and were used to inoculate a 50 litre jar fermentor which was held at 28 °C for 72 h.

The crude extract, obtained after usual work-up[5)] of the culture broth, was subjected to flash chromatography on silica gel with a MeOH/$CHCl_3$ gradient which gave a non polar semisolid fraction II containing mainly fats and fatty acids. In addition, the tlc of this fraction showed a relatively fast moving yellow band giving an intensive green fluorescence under 366 nm and the colour reactions which draw our attention in the crude extract. Further separation yielded 1.5 mg of parimycin as a light orange solid with all the above chemical and biological characteristics. The higher polar fractions contained a number of new trioxacarcine derivatives and gottingamycin which will be discussed elsewhere because of their completely different structures.

((Figure 1))

Results and discussion

Parimycin was obtained as a light orange solid which indicated relatively few signals in the ^1H NMR spectrum. It showed two typical signals of chelated OH groups at δ 14.36 and 13.36 supporting the assumption of being a *peri*-hydroxyquinone derivative. In the sp^2 region only two 1H singlets were visible. The aliphatic region delivered three singlets at δ 3.10 (2 CH_2), 2.98 (br, CH_3), 1.61 (CH_3), and the pattern of an ethyl residue.

The ^{13}C NMR spectrum of parimycin consisted of 22 signals of which according to the APT NMR spectrum fourteen signals represented quaternary carbons. In addition the

spectrum showed two methine, three methylene and three methyl carbons. The quaternary C signals at δ 201.6 and 201.0 could be assigned to α,β-unsaturated or aromatic ketones, one at δ 180.3 to the carbonyl carbon of a quinone or chromone system. The signal at δ 172.7 firstly pointed to the carbonyl carbon of an acid or ester, but was finally attributed to C-2 of a pyrone system as in AH-17 and AH-23[6] or the β-[7], γ- (**1**)[8] and δ-indomycinones[9]. The *sp*^{2} region delivered nine additional signals, where those at δ 156.7, 156.5 and 152.0 could be assigned to aromatic carbons connected to oxygen atoms. Furthermore, there was a signal at δ 73.6 for an aliphatic quaternary carbon connected to an oxygen atom. Between δ 7 – 36, signals of three methylene and three methyl groups were identified, thus confirming the results from the ^{1}H NMR spectrum.

((Table 1))

The (-)- and (+)-ESI spectra delivered quasi-molecular ions at *m/z* 395 ($[M-H]^+$) and 815 ($[2M+Na]^+$), respectively, which fixed the molecular weight to be 396 Dalton. High resolution measurement of the molecular ion afforded a molecular formula $C_{22}H_{20}O_7$ which was corresponding to the number of carbon and hydrogen atoms deduced from the ^{1}H and ^{13}C NMR spectra.

((Table 2))

((Figure 2))

The HMBC and H,H COSY couplings led to fragment I and the comparison of the NMR data with γ- (**1a**)[8] and δ-indomycinone[9] let the fragment I to be cyclized to fragment II which is also a substructure of γ-indomycinone (**1a**). The similarity of the shifts in substructures II and III of parimycin (**2**) and γ-indomycinone (**1a**), respectively, indeed supported the assumption of a close relation between them. The HMBC spectrum of parimycin showed couplings of the 4H singlet at δ 3.10 with ketone signals at δ 201.6 and 201.0, and additionally with aromatic C_q-signals at δ 110.0 and 108.5 which led to a second substructure IV. In the ^{1}H NMR spectrum two signals typical for strongly chelated hydroxy groups were observed which were responsible for the colour reaction with dilute sodium hydroxide. As only three carbonyl groups are present in the molecule, these data allowed substructure IV to be developed into V and ultimately to join II and V leading to structure **2** for parimycin.

((Figure 3))

((Formula 1/2))

Obviously parimycin (**2**) is the 2,3-dihydroquinone of the still unknown 1-hydroxy-γ-indomycinone (**1b**). Structurally related dihydro-quinones are easily obtainable by reduction of naphthazarine or quinizarine chromophores with tin(II)-chloride or by acidic rearrangement of hydroquinones[10]. 2,3-Dihydroquinizarine (**3**) obtained in this way from quinizarine (**4**) is a yellow compound, strongly green fluorescent similar to **2**. In alkaline solution it is re-oxidized immediately forming a violet solution of the **4**-anion.

((Figure 4))

Parimycin (**2**) gave the same reaction: When the natural product was treated with dilute sodium hydroxide and then neutralised with dilute hydrochloric acid followed by extraction with ethyl acetate, a pink solution presumably of **1b** with orange fluorescence at 366 nm resulted, which runs on tlc with a slightly higher R_f-value thus confirming the dihydroquinizarine chromophore.

In nature, 2,3-dihydroquinones are very rare. A search in AntiBase[11] and the Dictionary of Natural Products[12] delivered only 26 2,3-dihydro-1,4-quinones; only three of them were 2,3-unsubstituted, hortein[13] from a fungus and 2,3-dihydrojuglone[14] and 2,3-dihydro-diospyrin[15] from plants.

Biological Properties

Antibacterial, antimicroalgal and antifungal activities were qualitatively determined using the agar diffusion method. Parimycin (**2**) shows a similar activity to that of the related δ-indomycinone. At a concentration of >20 µg/disk, it was moderately active against *Bacillus subtilis* (BS), *Streptomyces viridochromogenes* (Tü 57), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC), however, inactive against *Mucor miehei* (TÜ 284), *Candida albicans*, and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* (Table 2). The higher activity of the crude extract in relation to the pure parimycin (**2**) is due to the content of trioxacarcins.

((Table 3))

In addition to the antibacterial activity, parimycin (**2**) showed anti-tumour activity against human tumor cell lines GXF 251L (stomach cancer), H460, LXFA 629L, and LXFL 529L (lung cancer models), MCF-7 and MAXF 401NL (breast cancer), MEXF 462NL and MEXF 514L (melanomas) with IC_{70} values ranging from 0.9 to 6.7 µg/ml.

Experimental

Material & methods and antimicrobial tests were used as described earlier[5].

Fermentation of *Streptomyces* sp. isolate B8652

The cell material from well grown agar plates of *Streptomyces* sp. isolate B8652 was used to inoculated 25 1 litre Erlenmeyer flasks shaking cultures, each containing 200 ml of yeast extract-malt extract medium[4]. The culture was grown for 3 days at 95 rpm and 28 °C and the broth was transferred under sterile condition into a 50 litre jar fermentor, containing 45 litres of the same medium as above and ca. 10 g Niax PPG 2025 (Union Carbide Belgium N.V. Zwijndrecht) to control foaming during the growth. Incubation was carried out at 28 °C for 3 days with supply of sterile air (5 l/min) and agitation of 120 rpm. 2 N NaOH and 2 N HCl were added automatically to maintain the pH at 6.50 ± 1.25.

The entire culture broth was mixed with ca. 1 kg celite, pressed through a pressure filter and both filtrate and residue were extracted with ethyl acetate to yield 16 g of crude extract containing ca. 10 g Niax. The extract was subjected to silica gel column chromatography (70 × 3 cm) using a $CHCl_3/CH_3OH$ step gradient (1300 ml $CHCl_3$ and each 1000 ml $CHCl_3$ with 1, 2, 3, 5, 7, 10, 13 % MeOH, then 300 ml $CHCl_3$/20 % MeOH and finally 200 ml MeOH as eluent) to give eight fractions (I, 3.23 g; II, 1.52 g; III, 1.57 g; IV, 9.65 g, mainly Niax; V, 630 mg; VI, 150 mg; VII, 150 mg; VIII, 660 mg). The active fraction II contained, in addition to the fat and fatty acids, a non polar yellow band with green fluorescence on tlc. It was then separated on Sephadex LH-20 (3 × 100 cm, MeOH) under tlc control into subfractions IIa (25 mg) and b. The later consisted mainly of fat and fatty acids and was not further analysed. The former containing the yellow zone on further separation by ptlc (20 × 20 cm, $CHCl_3$/5 % MeOH, developed twice) followed by final purification on Sephadex LH-20 (2 × 60 cm, $CHCl_3$/40 % MeOH) yielded 1.5 mg of parimycin (**2**) as a light orange solid. It forms on tlc a

yellow spot with green fluorescence at 366 nm. With anisaldehyde/sulphuric acid firstly an orange, then violet and finally pink colour is developed. For further data see tables 1 and 2.

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((figure 1))

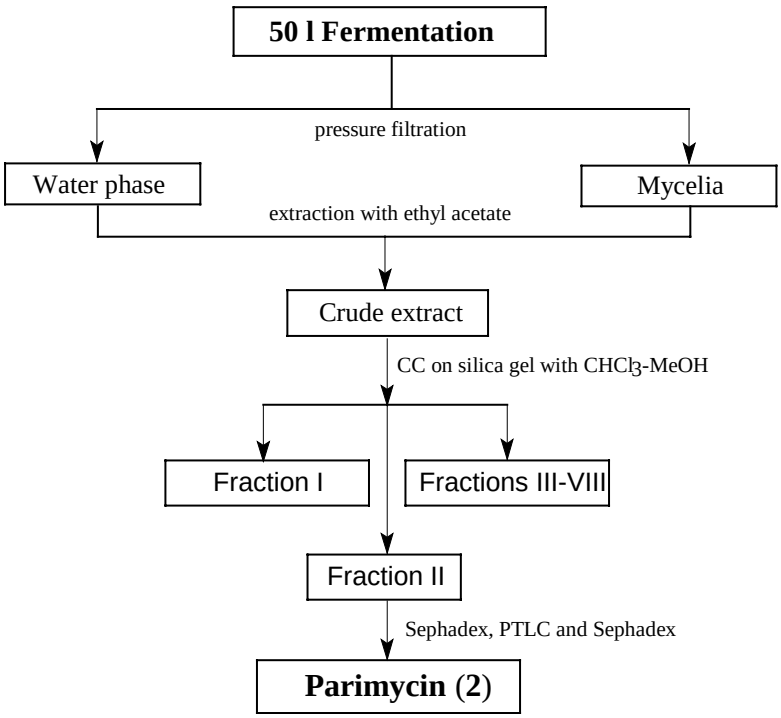
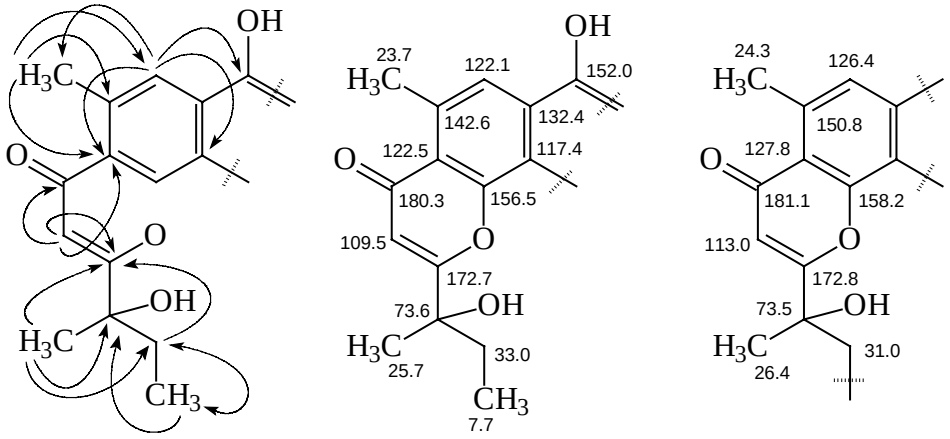


Figure 1. Working up scheme of the strain *Streptomyces* sp. isolate B8652

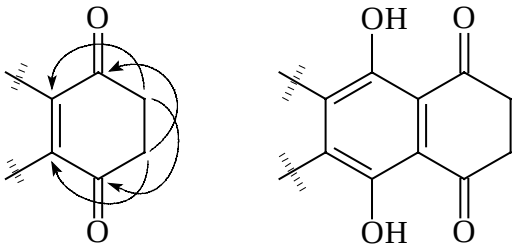
((Figure 2))



I **II** **III**

Figure 2. Substructure I constructed from HMBC (→) and H,H COSY (↔) couplings and developed to II by comparison of the NMR data with those of γ- and δ-indomycinone partial structure III

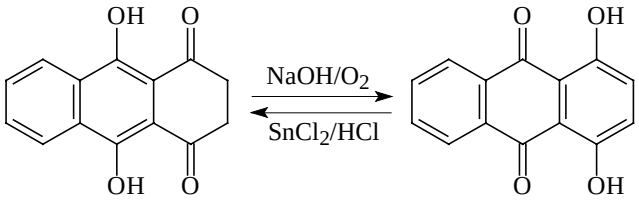
((Figure 3))



IV **V**

Figure 3. Substructures IV and V of parimycin constructed from 2D NMR data

((Figure 4))

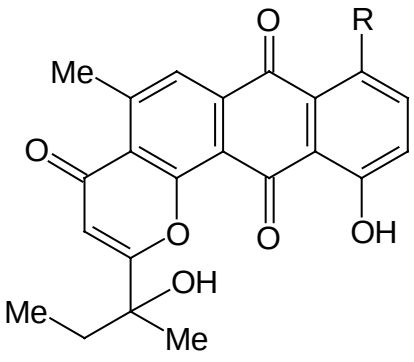


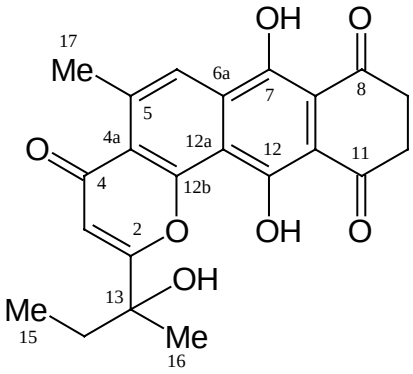
green fluorescence orange fluorescence

3 **4**

Figure 4. Oxidation of 2,3-dihydroquinizarin (**3**) to quinizarine (**4**) by air in the presence of alkali

((Formula 1/2)





1a: R = H **2**

1b: R = OH

Table 1. ^{13}C- ($CD_3OD/CDCl_3$) and ^{1}H-NMR ($CDCl_3$) data of Parimycin (**2**)

C-No.	Chemical shift (δ)		C-No.	Chemical shift (δ)	
	^{13}C-NMR	^{1}H-NMR		^{13}C-NMR	^{1}H-NMR
2	172.7	-	10	36.0[b]	3.10 (s)
3	109.5	6.53 (s)	11	201.6[a]	-
4	180.3	-	11a	108.5[c]	-
4a	122.5	-	12	156.7[d]	13.36[e] (s, OH)
5	142.6	-	12a	117.4	-
6	122.1	8.12 (s br)	12b	156.5[d]	-
6a	132.4	-	13	73.6	-
7	152.0	14.68[e] (s, OH)	14	33.0	2.05 (m), 1.92 (m)
7a	110.0[c]	-	15	7.7	0.93 (t, 6.8 Hz)
8	201.0[a]	-	16	25.7	1.61 (s)
9	35.8[b]	3.10 (s)	17	23.7	2.98 (s br)

[a-e] assignment may be reversed.

((Table 2))

Table 2. Physico-chemical Properties of Parimycin (**1**)

Properties	Light orange solid
R_f	0.52 ($CHCl_3$/10 % MeOH).
molecular formula	$C_{22}H_{20}O_7$
(+)-ESI-MS	815 ($[2M+Na]^+$)
(-)-ESI-MS	395 ($[M-H]^-$)
IR (KBr) ν (cm^{-1})	3450, 2925, 2885, 1637, 1630, 1465, 1412, 1388, 1321, 1259, 1180, 1110, 1000, 988, 799, 462
UV/VIS (MeOH): λ_{max} (lg ε)	260 (4.28), 423 (3.88), 447 (3.90) nm
$[\alpha]^{20}_D$	+60° (c 0.0309, MeOH)

((Table 3))

Table 3. Antibacterial activities of **2** (diameter of inhibition zones in mm).

	EC	BS	SV	SA
Crude extract	30	41	26	22
Parimycin	24	21	19	23