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Chalcomycin B, a New Macrolide Antibiotic from the Marine Isolate *Streptomyces* sp. B7064†

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In our screening of marine streptomycete isolates for bioactive components, a new macrolide antibiotic designated as chalcomycin B (**1b**) was isolated from the culture broth of a marine *Streptomycete* isolate B7064 together with chalcomycin (**1a**) as the active principles. The structure of the new antibiotic was determined by EI and ESI MS, ^{1}H, ^{13}C and 2D NMR spectroscopy and by comparison of the NMR data with those of chalcomycin.

Carbomycin[1], leucomycin[2], tylosin[3], spiramycin[4] and many other 16-membered macrolide antibiotics show potent activity against Gram-positive bacteria such as *Staphylococcus aureus*. This has stimulated the search for related substances and provided challenging targets in total synthesis of natural products. In the course of our screening program for novel bio-active compounds from marine Streptomycetes, the crude extract of the *Streptomyces* sp. B7064 drew our attention due to its high biological activity against a set of test organisms. In the chemical screening *semi*-polar zones on TLC were present which gave an unusual dark brown to black colouration with anisaldehyde/ sulphuric. The separation of the extract resulted in the isolation of chalcomycin (**1a**) and a new derivative chalcomycin B (**1b**) as the active principles, among with two simple known compounds, pyrrole-2-carboxylic acid methyl ester[5,6] and *N*-(2-phenylethyl)-acetamide[7]. In this paper we report on the taxonomy of the producing strain, as well as on the structure elucidation and the biological activity of **1b**.

Taxonomic Studies of the Producing Strain

Strain B7064 has been derived from mangrove sediment near Pohoiki, Hawaii (Pacific Ocean) and was isolated on chitin agar[8] containing 50% natural seawater. The reference culture of B7064 is kept on yeast extract-malt extract agar[8] in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven, Germany.

The almost complete16S rDNA gene sequence of the strain B7064 shows the highest similarity (98%) to the DNA of *Streptomyces peucetius* (strain ICM 9920). It is 97% similar to the DNA of *Streptomyces bikiniensis*.

The substrate mycelium is beige. Aerial mycelium is poorly developed with straight spore chains (*Rectiflexibiles*). The surface of spores is smooth. Melanin pigment is neither produced on peptone-yeast extract-iron agar nor on tyrosine agar[9]. Optimum growth temperature is at about 37°C. The strain does not grow at 45°C and 10°C and reproduces slowly at 18°C. Growth was not obtained in media with 7% or higher seawater salinity. Starch, casein, and chitin are degraded. Esculin and cellulose are not hydrolysed. The strain is catalase positive. Nitrate reductase and H_2S are not produced. The use of carbon sources was tested with SFN2-Biolog (Hayward, CA, USA) using BMS-N without agar as basal medium[10]. The following organic compounds can be utilized for growth: dextrin, glycogen, tween 40, tween 80, *N*-acetyl-D-glucosamine, D-galactose, D-glucose, maltose, D-mannose, D-melibiose, D-raffinose,

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D-trehalose, succinic acid, bromosuccinic acid, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, L-ornithine, L-phenylalanine, L-proline, L-serine, γ-amino butyric acid, urocanic acid, inosine, thymidine, 2-aminoethanol, and glycerol.

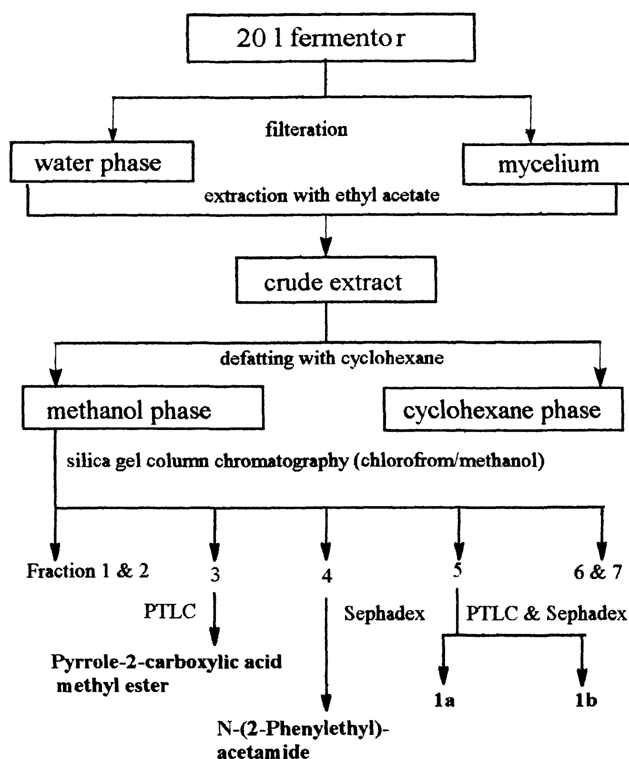
Fermentation and Isolation

Well-grown agar cultures of *Streptomyces* sp. B7064 served to inoculate ten 1 litre-Erlenmeyer flasks each containing 200 ml of yeast extract - malt extract medium[11]. The flasks were incubated with 95 rpm for 3 days at 28°C and were used to inseed a 20-litre jar fermentor which was held at 28°C for 72 hours (pH 6.5±1.25, 250 rpm). The crude extract, obtained after usual work-up[12] of the culture broth, was dissolved in methanol and defatted by shaking with cyclohexane.

A bio-activity guided fractionation of the defatted extract (flash silica gel column, chloroform/methanol gradient) using various test strains *viz. S. aureus*, *Mucor miehei*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Streptomyces viridochromogenes* (Tü 57), *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* delivered the active fraction 5 (see Fig. 1). This was further purified by PTLC and Sephadex LH 20 to afford chalcomycin B (**1b**), along with the known metabolite chalcomycin (**1a**). The structure of compound **1b** was assigned by a detailed interpretation of ^{1}H, ^{13}C, H,H-COSY, HMQC, HMBC, EI, and ESI mass spectra and further confirmed by the spectral similarity with chalcomycin (**1a**).

Two UV absorbing bands in fractions 3 and 4 yielded the frequently occurring derivatives pyrrole-2-carboxylic acid methyl ester and *N*-(2-phenylethyl)-acetamide.

Fig. 1. Separation of extracts from the marine *Streptomycete* isolate B7064.



Results and Discussion

Compound A was obtained from fraction 5 as a colourless solid with peaks at m/z=723.4 ([M+Na]$^+$) and 1422.7 ([2M+Na]$^+$) in the (+)-ESI mass spectrum, corresponding to a molecular weight of 700 Da.

The ^{1}H-NMR spectrum showed in the olefinic region a 1H dd signal at δ=6.66 (^3J=15.4 and 10.2 Hz), one 2H-multiplet at δ=6.58 and one doublet at δ=5.82 (^3J=15.4 Hz). A further quartet of a doublet with intensity 1 at δ=5.34, two 1H doublets at δ=4.58 and 4.22, many 1H multiplets typical for sugar residues between δ=4.0 and 3.0 and three methoxy signals at δ=3.63, 3.57 and 3.42 were identified. Additionally, the spectrum showed multiplets at δ=2.72 (1H), 2.05 (1H) and 1.91 (2H), one methyl singlet at δ=1.39 and five methyl doublets at δ=1.35, 1.28, 1.23, 1.21 and 1.01.

The ^{13}C-NMR spectrum indicated a ketone signal at δ=200.2 which must be conjugated with a double bond because of its chemical shift. A further carbonyl signal at δ=165.3 represented either a carboxylic acid, an ester or an amide. Four olefinic methine signals in the sp^2-region at δ=151.8, 146.5, 124.8 and 120.7 could be interpreted as two double bonds conjugated with carbonyl groups. The methine signals at δ=103.2 and 100.9 were interpreted as due to two acetal carbon atoms. In addition to these signals, 16 signals of carbon atoms connected to oxygen between δ=87.8~56.7, CH-signals at δ=49.5, 41.7 and 34.0, methylene-signals at δ=37.0 and 36.8 and six methyl signals in the range δ=27.8~17.8 were observed.

The search in AntiBase[13] with the above mentioned spectral information led to chalcomycin (**1a**), a 16-membered macrolide previously reported from *S. bikiniensis*[14]. The structure was confirmed by direct comparison of the NMR data[15].

A second compound B was obtained as well from fraction 5 as a colourless solid. The ^{1}H NMR spectrum showed a very close similarity in the range of δ=6.6~5.3

Table 1. ^{13}C NMR data of chalcomycin A (**1a**) and chalcomycin B (**1b**) in deuteriochloroform (125 MHz).

C-Atom	Chemical shift (δ)		C-Atom	Chemical shift (δ)		C-Atom	Chemical shift (δ)	
	1a	**1b**		**1a**	**1b**		**1a**	**1b**
1	165.3	165.3	15	68.9	68.8	8'	-	27.7
2	120.7	120.9	16	18.6	18.2	9'	-	9.1
3	151.8	151.3	17	19.2	19.1	1"	100.9	100.8
4	41.7	41.3	18	27.8	27.8	2"	81.9	80.1
5	87.8	88.5	19	66.9	67.1	2"-OMe	58.7	59.4
6	34.0	34.0	20	18.3	18.3	3"	79.6	77.6
7	37.0	37.1	1'	103.2	101.6	3"-OMe	61.7	61.6
8	78.4	78.4	2'	75.0	74.2	4"	72.9	74.6
9	200.2	200.0	3'	80.5	78.8	5"	70.7	67.4
10	124.8	125.0	3'-OMe	56.7	56.5	6"	17.8	17.4
11	146.5	146.3	4'	36.8	37.1	7"	-	173.6
12	59.7	58.7	5'	67.7	67.7	8"	-	27.6
13	59.0	59.0	6'	20.9	20.8	9"	-	9.2
14	49.5	49.4	7'	-	173.2			

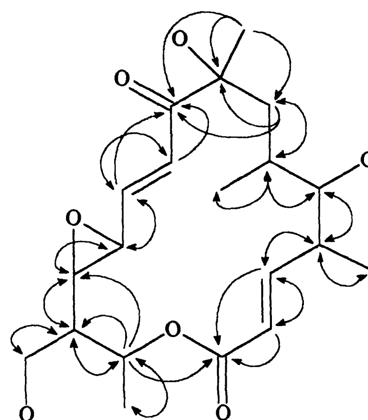
with that of chalcomycin (**1a**). In the region typical for sugar residues several signals were present but showed different chemical shifts as compared to **1a**. Furthermore, signals of eight methyl groups connected to sp^3-carbons, namely a singlet, five doublets and two triplets between $\delta=1.40\sim0.90$, and three methoxy signals at $\delta=3.48$, 3.46 und 3.26 were visible. Additionally to the spectrum of chalcomycin (**1a**), a 4H multiplet for two methylene groups connected to carbonyl groups at $\delta=2.36\sim2.23$ and two methyl triplets at $\delta=1.10$ and 1.12 were present, indicating two propionyl groups.

The ^{13}C NMR and APT spectra of **1b** revealed the presence of 41 carbons. A comparison of the ^{13}C NMR data of chalcomycin (**1a**) with those of compound B (Table 1) indicated six additional carbon signals, which appeared as twin resonances at $\delta=173.6/173.2$, 27.7/27.6 and 9.2/9.1. This and the splitting pattern of the extra methyl signals indicated that compound B could be an **1a** dipropionate.

This was supported by the (+)-ESI mass spectra with quasi molecular peaks at $m/z=1647$ (2M+Na) and 835 (M+Na), corresponding to a molecular weight of 812 which is $\Delta m=112$ Dalton ($\equiv 2$ CH_3CH_2CO) higher than that of chalcomycin (**1a**).

The structure of compound B was finally derived by the interpretation of 2D NMR spectra namely H,H-COSY, HMQC and HMBC spectra. With the aid of 2D couplings from H,H-COSY, HMQC (not shown in Fig. 2) and HMBC spectra and the coupling constants of the proton signals in the ^1H NMR spectrum, the aglycon (Fig. 2) was constructed, which was identical with that of chalcomycin (**1a**).

Fig. 2. H,H-COSY (↔) and HMBC (→) couplings in the aglycon of chalcomycin B (**1b**).



Likewise the connectivity of two sugar residue was derived with the aid of 2D couplings of H,H-COSY, HMQC and HMBC spectra (Figs. 3 and 4) and the relative configuration was established through the coupling constants of the adjacent proton signals. The sugar residues were found to be chalcose-2-propionate (Fig. 3) and 6-deoxy-2,3-di-*O*-methylallose-4-propionate (Fig. 4), *i.e.* the mono propionates of the sugar residues of chalcomycin (**1a**). Correspondingly the configuration should be D in both residues.

The link of the sugar residues with the aglycone was determined by the HMBC couplings of the anomeric proton with the respective carbon atom of the central unit. The anomeric proton of D-chalcose-2-propionate at $\delta=4.22$ couples with C-5 ($\delta=88.5$) and that of D-6-deoxy-2,3-di-*O*-methylallose-4-propionate at $\delta=4.57$ with C-20 ($\delta=67.1$) of the aglycon, which confirmed the structure of the compound B to be **1b**. We suggest to name it as chalcomycin B and propose chalcomycin (**1a**) to be renamed as chalcomycin A.

Antibacterial Activity

Antibacterial and antifungal activities were qualitatively determined using the agar diffusion method. Activities on *B. subtilis* (BS), *S. viridochromogenes* (Tü 57), *S. aureus* (SA), *E. coli* (EC), *C. albicans* (CA) and *M. miehei* (MM) as test organisms are listed in Table 1, the MIC values against some micro-organisms and micro-algae are summarized in Table 2. Both compounds show identical activities with emphasis on *S. aureus*. The phytotoxic activity of **1a** had not been reported previously, however, is low as in **1b**.

Experimental

Material and methods and antimicrobial tests were used as described earlier[12].

Fig. 3. H-H COSY (↔) and HMBC (→) couplings in the D-chalcose-2-propionate moiety of chalcomycin B (**1b**).

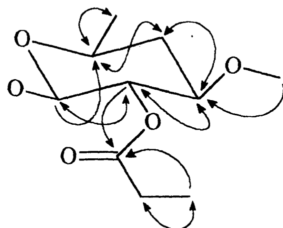
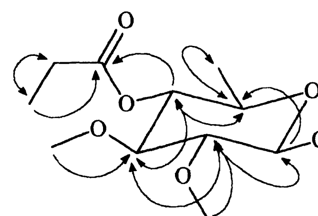
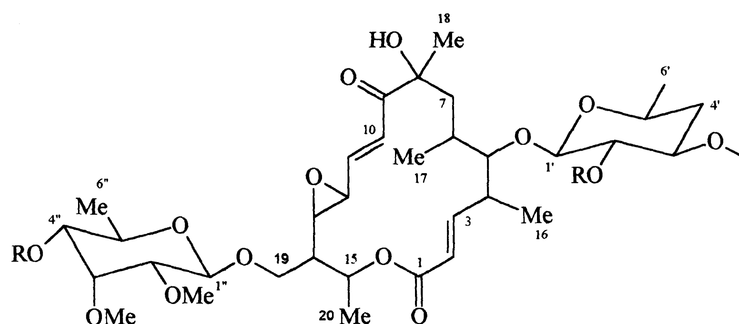


Fig. 4. H-H COSY (↔) and HMBC (→) couplings in the D-6-deoxy-2,3-di-*O*-methylallose-4-propionate unit of chalcomycin B (**1b**).



Structure 1.



1a: R = H

1b:R = EtCO

Table 2. Physico-chemical properties of chalcomycins A and B (**1a** and **1b**).

	Chalcomycin A (**1a**)	Chalcomycin B (**1b**)
State	colourless solid	colourless solid
R_f	0.55[a]	0.78[a]; 0.60[b]
Molecular formula	$C_{35}H_{56}O_{14}$	$C_{41}H_{64}O_{16}$
M. P.	120-121 °C	98 °C
(+)-ESI-MS	723 ([M+Na]$^+$), 1423 ([2M+Na]$^+$)	835 ([M+Na]$^+$), 1647 ([2M+Na]$^+$)
ESI-HRMS		calcd. 812.4194 found 812.4191
IR (KBr) ν cm^{-1}	3490, 2930, 1718, 1630, 1498, 1350, 1236, 1170, 1083, 982, 890, 726	3445, 2935, 1740, 1730, 1634, 1459, 1355, 1178, 1080, 980, 850, 804

[a]$CHCl_3$/10 % MeOH, [b]$CHCl_3$/5 % MeOH

Table 3. Antimicrobial activities of **1a**, **1b**, and erythromycin (**E**) in the agar diffusion test (diameter of inhibition zone in mm at 10 μg/9 mm i.d. platelet).

	SA[a]	EC[b]	BS[c]	CA[d]	MM[e]
1a	30	32	25	0	0
1b	23	28	21	0	0
E	24	30	22	-	-

[a] *Staphylococcus aureus*, [b] *Escherichia coli*, [c] *Bacillus subtilis*, [d] *Candida albicans*, [e] *Mucor miehei*.

Table 4. Antimicrobial activity of **1a** and **1b** by serial dilution method; MIC (μg /ml).

	SA	EC	BS	CV[a]	CS[b]	SS[c]
1a	0.39	>50	6.25	50	50	50
1b	0.39	>50	6.25	50	50	50

[a] *Chlorella vulgaris*, [b] *Chlorella sorokiniana*, [c] *Scenedesmus subspicatus*.

Fermentation of the Isolate *Streptomyces* sp. B7064

The marine strain *Streptomyces* sp. B7064 was grown for 3 days at 95 rpm and 28°C in ten 1 litre Erlenmeyer flasks, each containing 200 ml of yeast extract-malt extract medium[11]. The broth was transferred into a 20-litre jar fermentor, containing 18 litres of the same medium as above. Incubation was carried out at 28°C for 3 days with supply of sterile air (5 litres/minute) and agitation of 120 rpm. Niax and 2 N NaOH were added automatically to control foaming and to maintain the pH at 6.50±1.25.

The entire culture broth was extracted with ethyl acetate to yield 2.6 g of crude extract which was defatted with cyclohexane. The crude extract (1.95 g) was subjected to silica gel column chromatography (65×3 cm) using a $CHCl_3/CH_3OH$ gradient (7 steps, 1~10% MeOH, each 500 ml) as eluent to give seven fraction. The active fraction 5 was separated by PTLC ($CHCl_3/CH_3OH$, 9 : 1) to provide two compounds which were further purified on Sephadex LH 20 (80×3 cm, MeOH) to yield chalcomycin (**1a**, 14 mg) and chalcomycin B (**1b**, 7.8 mg). The third fraction gave on PTLC ($CHCl_3/CH_3OH$, 19 : 1) pyrrole-1-carboxylic acid methyl ester (12 mg), while the fourth fraction afforded on Sephadex LH 20 separation *N*-(2′-phenylethyl) acetamide (25.9 mg).

Chalcomycin B (**1b**)

$^{20}[\alpha]_D = +186$ (c=5.900 mg/100 ml, MeOH). - ^{1}H-NMR ($CDCl_3$, 500 MHz): δ=6.57 (dd, ^3J=15.6, 10.1 Hz, 1H, 3-H), 6.51 (m, 2H, 10-H, 11-H), 5.74 (d, 15.6 Hz, 1H, 2-H), 5.32 (dq, ^3J=10.7, 6.3 Hz, 1H, 15-H), 4.76 (dd, ^3J=9.4, 7.8 Hz, 1H, 2′-H), 4.58 (d, ^3J=8.0 Hz, 1H, 1″-H), 4.41 (dd, ^3J=9.9, 2.5 Hz, 1H, 4″-H), 4.22 (d, ^3J=8.0 Hz, 1H, 1′-H), 4.12 (dd, ^2J=10.3 Hz, ^3J=3.2 Hz, 1H, 19-H_A), 3.90~3.84 (m, 2H, 3″-H, 5″-H), 3.62 (dd, ^2J=10.3 Hz, ^3J=3.2 Hz, 1H, 19-H_B), 3.48 (s, 3H, 2″-OCH_3), 3.46 (s, 3H, 3″-OCH_3), 3.42 (m, 1H, 5′-H), 3.27 (m, 1H, 3′-H), 3.26 (s, 3H, 3′-OCH_3), 3.25 (m, 1H, 12-H), 3.10 (dd, ^3J=9.1, 2.0 Hz, 1H, 13-H), 3.05 (d, ^3J=9.1 Hz, 1H, 5-H), 3.03 (dd, ^3J=8.1, 3.0 Hz, 1H, 2″-H), 2.50 (m, 1H, 4-H), 2.36~2.23 (m, 4H, 8′-H_2, 8″-H_2), 2.01 (m, 1H, 4′-H_A), 1.88 (dd, ^2J=14.9 Hz, ^3J=12.2 Hz, 1H, 7-H_A), 1.76 (dd, ^2J=14.8 Hz, ^2J=3.2 Hz, 1H, 7-H_B), 1.36 (m, 2H, 4′-H_A, 14-H), 1.31 (s, 3H, 18-H_3), 1.28 (d, ^3J=6.4 Hz, 3H, 20-H_3), 1.20 (d, ^3J=6.4 Hz, 3H, 6′-H_3), 1.20 (m, 1H, 6-H), 1.12 (t, ^3J=7.4 Hz, 3H, 9′-H_3), 1.11 (d, ^3J=6.2 Hz, 3H, 6″-H_3), 1.10 (t, ^3J=7.3 Hz, 3H, 9″-H_3), 1.02 (d, ^3J=6.6 Hz, 3H, 16-H_3), 0.94 (d, ^3J=6.9 Hz, 3H, 17-H_3). - EI-MS: m/z=812 (M, 2), 595 (3), 565 (17), 509 (1), 349 (3), 231 (47), 201 (83), 169 (100), 143 (5), 88 (35), 57 (23).

Acknowledgements

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