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Chandrananimycins A~C: Production of Novel Anticancer Antibiotics from a Marine *Actinomadura* sp. Isolate M048 by Variation of Medium Composition and Growth Conditions†

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In our screening of marine actinomycetes for bioactive principles, three novel antibiotics designated as chandrananimycin A (**3c**), B (**3d**) and C (**4**) were isolated from the culture broth of a marine *Actinomadura* sp. isolate M045. The structures of the new antibiotics were determined by detailed interpretation of mass, 1D and 2D NMR spectra.

In the course of our screening program for bioactive principles from marine actinomycetes, the crude extract of *Actinomadura* sp. isolate M048 drew our attention due to its high biological activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptomyces viridochromogenes* (Tü 57). It also exhibited antialgal activity against the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and had antifungal activity against *Mucor miehei*. The chemical screening of the extract showed middle polar to non-polar yellow and red zones which were found to be responsible for the observed antimicrobial activity. Work-up of the extract resulted in the isolation of three new antibiotics with the phenoxazin-3-one skeleton, chandrananimycin A (**3c**), B (**3d**), and C (**4**).

Compounds containing the phenoxazin-3-one chromophore are frequently encountered as metabolites of microorganisms. They are yellow to orange coloured compounds and exhibit antibacterial[1], antifungal[2], phytotoxic[3,4] and anticancer[5] activities. In addition, some are also known to possess potent cell-growth stimulating activity[6]. The chromophore is part of complex natural products like actinomycin, aurantin and cryptomycin and is responsible for their colour. Due to their DNA intercalation, these complex phenoxazinone derivatives are showing pronounced antimicrobial[7] and anticancer potency[8] as well, and some of them also exhibit anticoccidial activity[9]. In this paper we report the taxonomy of the producing strain, the isolation, structure elucidation and the biological activity of the new natural products.

Taxonomy of the Producing Strain

The strain M048 has been derived from the sediment of Jiaozhou Bay in China and was isolated on Gause's starch medium while incubating at 28°C. The pure culture was maintained on Gause's starch agar medium with $K_2Cr_2O_7$ at 4°C. The strain forms lilaceous vegetative mycelium which has branches but does not break and white aerial mycelium which always breaks. The strain forms spiral spore chains (*Spirales*). Melanin or other water-soluble pigments were not produced on tyrosine agar[10]. The temperature optimum is at approximately 28°C. The strain does not grow at 45°C or at 10°C. Gelatine and starch are degraded. Hydrogen sulphide is not produced. The strain is catalase positive and nitrate reductase is not formed. The peptidoglycan cell wall of the strain contains major amounts of *meso*-diaminopimelic acid (*meso*-DAP) and madurose, but no glycine in cell wall hydrolysates of the strain (cell wall

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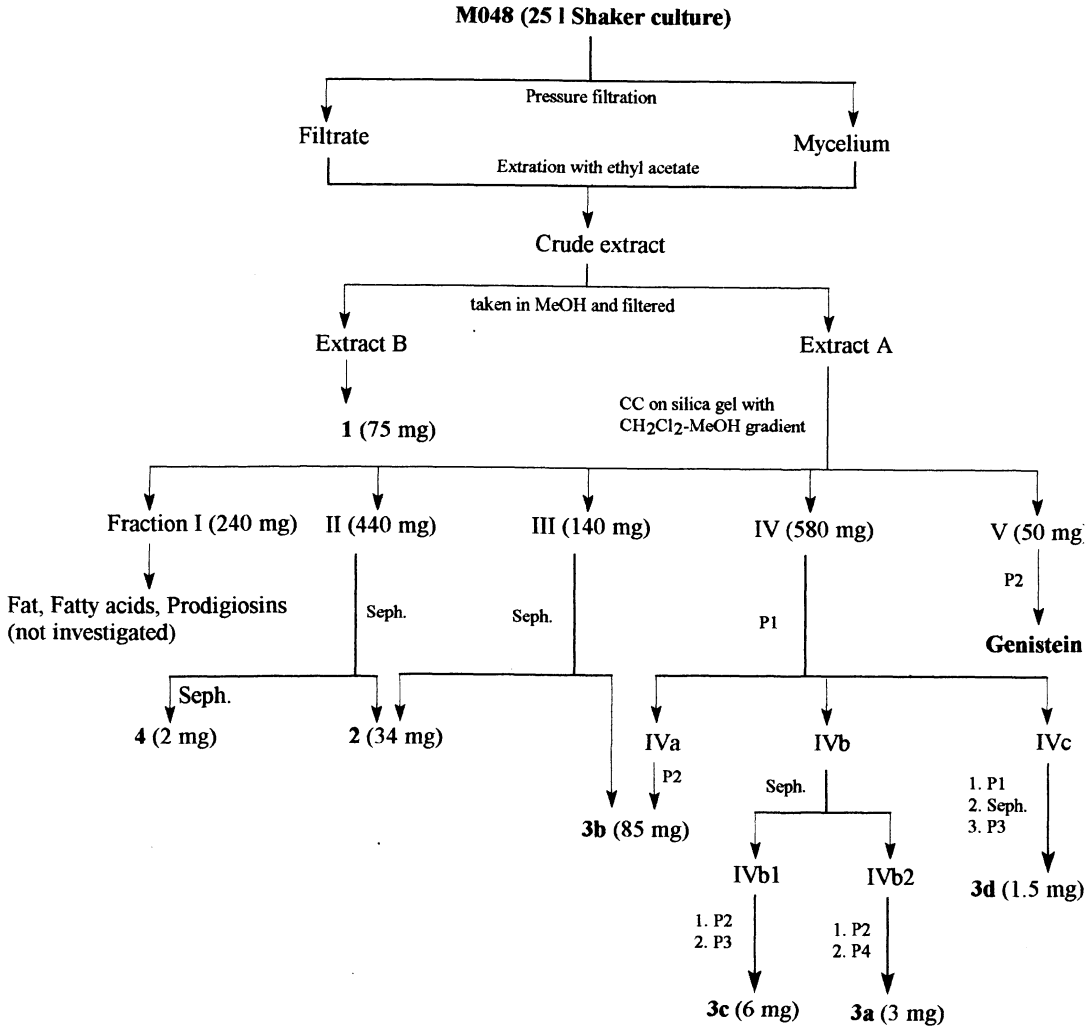
Fig. 1. Working-up scheme of the strain *Actinomadura* sp. isolate M048.



Seph.=CC on Sephadex LH 20 (CH_2Cl_2/60% MeOH), P1=PTLC (CH_2Cl_2/5% MeOH), P2=PTLC (CH_2Cl_2/3% MeOH), P3=PTLC (CH_2Cl_2/10% acetone) and P4=PTLC (CH_2Cl_2/20% acetone).

chemotype III)[10]. Due to its chemical and morphological features the strain can be assigned to the genus *Actinomadura*. The strain with the number M048 is deposited in the culture collection of marine actinomycetes at the Institute of Oceanology, Chinese Academy of Sciences, Nanhai Road 7, 266071 Qingdao, China.

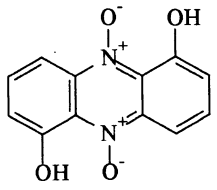
Fermentation and Isolation

On incubation on M_2 agar at 28°C for 72 hours, the *Actinomadura* sp. isolate M048 formed a lilaceous vegetative and white aerial mycelium. With a well grown agar culture, 100 1-liter-Erlenmeyer flasks each containing 250 ml of SM medium were inoculated and kept at 28°C for 4 days while shaking with 95 rpm. The crude extract, obtained after usual work-up of the culture broth[12], was repeatedly separated on silica gel, delivering six known metabolites, namely iodinin (**1**)[13], 1,6-phenazinediol (**2**)[14], questiomycin A (**3a**)[15], *N*-acetylquestiomycin A (**3b**)[15], genistein[16,17], daidzein[18], and three new antibiotics chandrananimycin A (**3c**), B (**3d**), and C (**4**).

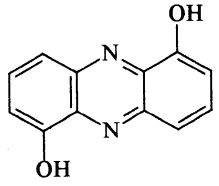
Results and Discussion

Compounds **1** and **2** were obtained as violet and yellow solids, respectively. By CI and EI mass spectrometry, the molecular weights were fixed to be *m/z* 244 (**1**) and 212 (**2**).

Formula **1**, **2**

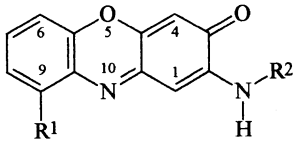


1

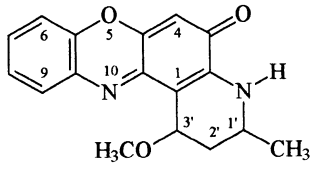


2

Formula **3**, **4**



3a: $R^1 = R^2 = H$
3b: $R^1 = H$; $R^2 = COCH_3$
3c: $R^1 = OH$; $R^2 = COCH_3$
3d: $R^1 = H$; $R^2 = COCH_2OH$



4

The ^1H NMR spectra showed only three aromatic ABC signals of equal intensity and the ^{13}C NMR spectra exhibited six signals for both compounds. The search with these spectroscopic data in AntiBase[19] led to iodinin (**1**) and 1,6-phenazinol (**2**), and the structures were finally confirmed by comparison of the NMR data with the literature values[13,14].

Compounds **3a**~**d** and **4** were obtained as orange solids. The missing colour reaction with dilute sodium hydroxide, a red colouration with concentrated sulphuric acid, a deep orange colouration with anisaldehyde/sulphuric acid and the similarity of the UV/VIS spectra with the data of actinomycins indicated they contain phenoxazinone chromophores, which were often found during our screening of actinomycetes.

ESI MS did not afford molecular ions, however, by CI MS, the molecular weights of the compounds were determined to be *m/z* 212 and 254, respectively, for compounds **3a** and **3b**. The ^1H NMR spectrum of compound **3a** showed six sp^2 proton signals, four consecutive vicinal protons of a benzene ring at δ 7.70~7.38 and two olefinic protons at δ 6.37 and 6.35. Additionally, a H/D-exchangeable signal of a amino group was observed at δ 6.76. The proton NMR spectrum of **3b** was very much similar to that of **3a**, except that one of the olefinic proton signals was shifted downfield, there was only one acidic proton and an additional acetate methyl signal was observed. By the similarity in the NMR data and the mass difference of Δm 42, **3a** and **3b** were easily identified as questiomycin A[15,20] and *N*-acetylquestiomycin A[15,21]. According to the 2D data, however, the signal assignments of C-5a and C-10a in **3b** are corrected.

The molecular weight of **3c** was determined as *m/z* 270 by CI and EI MS, and EI HRMS led to the molecular formula $C_{14}H_{10}N_2O_4$. The only difference between **3b** and **3c** is the substitution of one of *peri*-proton of the benzene ring in **3b** by a hydroxyl group in **3c** which is expressed by the difference in the formula and by the signal pattern of three instead of four consecutive vicinal aromatic protons. While the ^{13}C signals of C-5a, 6 and 7 remained nearly unchanged with respect to the signals of **3b** ($\Delta\delta$ 0.5~2.5), C-8 and C-9a are strongly high-field shifted ($\Delta\delta$ 10~20). The OH group must be therefore located at C-9.

The molecular formula $C_{14}H_{10}N_2O_4$ of **3d** derived from the EI HRMS of the molecular ion peak at *m/z* 270 shows that **3d** must be a *mono*-hydroxyl derivative of **3b**. In the ^1H NMR spectrum of **3d**, the acetate methyl signal of **3b** was replaced by a CH_2 signal at δ 4.10, the corresponding carbon signal was downfield shifted to δ 61.8. As all sp^2 carbon and proton signals were nearly identical with those of **3b**, **3d** is the glycolate of **3a**.

The molecular weight of **4** was determined to be *m/z* 296 and EI HRMS delivered the molecular formula $C_{17}H_{16}N_2O_4$. The ^1H NMR spectrum of **4** exhibited signals for four consecutive aromatic protons and an olefinic proton signal at δ 6.35. As compared to that of **3b**, one singlet in olefinic region at δ 8.26 and the acetate methyl signal at δ 2.22 were missing in the spectrum of **4**. But it depicted aliphatic proton signals of a methoxy, a methyl group in direct neighbourhood to a methine group, a methylene and two methine groups which were not present in the spectra of **3a**~**3d**. The structure **4** was determined by careful interpretation of mass and NMR spectra and by comparison of the NMR data and molecular formula with those of **3a**~**3d** and finally confirmed by 2D NMR measurements.

Biological Properties

Antibacterial against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, antifungal against *Mucor miehei* and *Candida albicans*, and antialgal activities

Table 1. ^{1}H NMR (DMSO-d_6, 500 MHz) data of chandrananimycins A (**3c**), B (**3d**) and C (**4**) (δ values, J [Hz]).

H No.	3a	3b[a]	3c	3d[b]	4[c]
1-H	6.37 (s)	8.26 (s)	8.35 (s)	8.26 (s)	-
4-H	6.35 (s)	6.47 (s)	6.41 (s)	6.54 (s)	6.35 (s)
6-H	7.47 (d, 7.6)	7.55 (d, 7.5)	6.86 (d, 8.3)	7.49 (d, 7.7)	7.35 (m)
7-H	7.45 (t, 7.6)	7.63 (t, 7.5)	7.41 (t, 8.3)	7.66 (t, 7.7)	7.35 (m)
8-H	7.38 (t, 7.6)	7.47 (t, 7.5)	6.90 (d, 8.3)	7.58 (t, 7.7)	7.35 (m)
9-H	7.70 (d, 7.6)	7.85 (d, 7.5)	-	7.89 (d, 7.7)	7.76 (dd, 7.7, 1.5)
$COCH_3$	-	2.22 (s)	2.22 (s)	-	-
$COCH_2$	-	-	-	4.10 (s)	-
NH	-	9.67 (s)	9.59 (s)	9.76 (s)	5.88 (s br)
NH_2	6.76 (s br)	-	-	-	-
OH	-	-	-	6.21 (s br)	-
1'-H	-	-	-	-	3.73 (m)
2'-H_2	-	-	-	-	2.22 (m), 1.59 (m)
3'-H	-	-	-	-	5.07 (t, 2.7)
1'-CH_3	-	-	-	-	1.38 (d, 6.5)
OCH_3	-	-	-	-	3.54 (s)

[a]300 MHz, [b]600 MHz, [c]$CDCl_3$

Table 2. ^{13}C NMR (DMSO-d_6, 150.8 MHz) data of chandrananimycin A (**3c**), B (**3d**) and C (**4**) (δ values).

C No.	3a	3b[a]	3c[b]	3d	4[a]	C No.	3a	3b[a]	3c[b]	3d	4[a]
1	98.4	113.8	111.8	112.7	105.1	9°	133.7	134.0	124.1	133.4	134.0
2	148.8	148.8	148.8	148.4	146.3	10a	147.3	137.2	137.0	137.0	142.5
3	180.1	179.7	179.1	179.0	179.9	NCO	-	169.2	170.4	172.0	-
4	103.4	104.0	103.6	103.6	103.7	$COCH_3$	-	24.9	24.3	-	-
4a	148.2	149.4	155.7[c]	149.2	150.0	CH_2OH	-	-	-	61.8	-
5a	141.9	143.2	143.7	142.9	142.4	1'	-	-	-	-	42.2
6	115.8	116.1	113.6	116.1	115.9	2'	-	-	-	-	33.8
7	128.7	131.8	132.4	132.1	128.9	3'	-	-	-	-	67.8
8	125.2	125.7	105.4	125.7	125.1	1'-CH_3	-	-	-	-	20.9
9	127.9	130.1	145.2[c]	129.5	128.8	OCH_3	-	-	-	-	56.7

[a]$CDCl_3$, [b]125.7 MHz, [c]assignment may be exchanged

Table 3. Physico-chemical properties of chandrananimycins A (**3c**), B (**3d**) and C (**4**).

	3c	**3d**	**4**
Properties	orange solid	orange solid	Orange solid
R_f	0.45	0.21	0.29
Molecular formula	$C_{14}H_{10}N_2O_4$	$C_{14}H_{10}N_2O_4$	$C_{17}H_{16}N_2O_3$
CI-MS (NH_3)	271 ($[M+H]^+$)	271 ($[M+H]^+$)	297 ($[M+H]^+$)
IR (KBr) ν cm^{-1}	3406, 3336, 2924, 2853, 1698, 1647, 1615, 1586, 1514, 1485, 1444, 1346, 1280, 1249, 1169, 1144, 1060, 880, 848, 788, 751, 681, 593, 510, 462	3427, 2921, 2852, 1620, 1579, 1520, 1462, 1438, 1421, 1405, 1383, 1349, 1263, 1172, 1074, 956, 882, 849, 765, 745, 542, 468	3426, 2923, 2851, 1636, 1575, 1516, 1457, 1373, 1311, 1281, 1206, 1107, 1033, 998, 888, 856, 785, 763, 672, 579, 532, 472
UV/VIS (MeOH): λ_{max} (lg ε)	226 (4.52), 270 (4.38), 423 (4.45)	238 (3.85), 399 (3.66)	243 (4.49), 282 (4.19), 366 (4.16), 433 (4.20)

Table 4. Antimicrobial activities of **1**, **2**, **3a**~**d**, and **4** (20 μg/platelet, diameter of inhibition zones in [mm]).

	EC	BS	SA	MM	CA	CV	CS	SS
Iodinin (**1**)	0	12	16	11	0	0	0	0
1,6-Phenazinediol (**2**)	0	15	15	11	0	0	0	0
Questiomycin A (**3a**)	0	12	0	23	0	11	12	13
N-Acetylquestiomycin A (**3b**)	0	0	0	12	14	0	0	0
Chandrananimycin A (**3c**)	0	12	0	11	0	0	0	0
Chandrananimycin B (**3d**)	0	0	0	12	0	0	0	0
Chandrananimycin C (**4**)	0	23	22	27	0	11	11	15

EC = *Escherichia coli*, BS = *Bacillus subtilis*, SA = *Staphylococcus aureus*, MM = *Mucor miehei*, CA = *Candida albicans*, CV = *Chlorella vulgaris*, CS = *Chlorella sorokiniana*, SS = *Scenedesmus suspicatus*

against the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus suspicatus* were *semi*-quantitatively determined using the agar diffusion method.

Biological activities of the compounds can be considered to be moderate to strong, depending on the structures. Except in case of questiomycin A (**3a**) and chandrananimycin C (**4**), they exhibited comparable activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Mucor miehei*. It is remarkable that **3a** and **4** not only showed strong activity against *Mucor miehei* but also antialgal activity, which was not reported for this group so far. Chandrananimycin A (**3c**) showed weak activity similar to that of iodinin, however, chandrananimycin C (**4**) presented the strongest activity against *Bacillus subtilis* and *Staphylococcus aureus* in this group. None of the tested compounds was active against *Escherichia coli*.

The compounds **3a**~**d** and **4** were also found to be active against the human cell lines, CCL HT29 (colon carcinoma), MEXF 514L (melanoma), LXFA 526L (lung carcinoma), LXFL 529L (lung carcinoma), CNCL SF268, LCL H460, MACL MCF-7 (breast carcinoma), PRCL PC3M, RXF 631L (kidney tumor) with IC_{70} values down to 1.4 μg/ml.

Iodinin (**1**) and 1,6-phenazinediol (**2**) also exhibited antitumour activity against the human cell lines LXFA 629L and LXFL 529L (lung), MAXF 401NL (breast), MEXF 462NL (melanoma), RXF 944L (kidney) and UXF 1138L (uterus) with IC_{50} values of 3.6 and 3.2 μg/ml, respectively.

Experimental

Materials, methods and antimicrobial tests were used as described earlier[12]. Rf values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.) with dichloromethane/3% methanol when not stated otherwise.

M_2 Medium

Malt extract (10 g), yeast extract (4 g) and glucose (4 g) were dissolved in artificial sea water (0.5 liters) and tap water (0.5 liters). Before sterilisation, the pH was adjusted to 7.8 by addition of 2 N NaOH.

SM Medium

Soy bean flour (20 g) and mannitol (20 g) were dissolved in artificial sea water (0.5 liters) and tap water (0.5 liters).

ME Medium

Meat extract (1 g), glucose (10 g), peptone (2 g) and yeast extract (1 g) were dissolved in artificial sea water (0.5 liters) and tap water (0.5 liters).

LB Medium

Tryptone (10 g), yeast extract (5 g), sodium chloride (10 g) and glucose (5 g) were dissolved in artificial sea water (0.5 liters) and tap water (0.5 liters).

FF Medium

Glucose (21 g), fish powder (5 g), wheat flour (10 g), yeast extract (1 g), magnesium sulphate (0.5 g), sodium chloride (1 g), calcium chloride (0.5 g) and trace element solution (10 ml) were dissolved in artificial sea water (0.5 liters) and tap water (0.5 liters); trace element solution: Ferrous sulphate (10.2 g), cobalt chloride (0.04 g), calcium chloride (0.04 g), manganese chloride (0.04 g), zinc sulphate (0.08 g), sodium borate (0.08 g), and sodium molybdate (0.74 g) were dissolved in 500 ml of dest. water.

Gause's Starch Medium

Soluble starch (20 g), potassium nitrate (1 g), potassium hydrogen phosphate (0.5 g), magnesium sulphate (0.5 g), sodium chloride (0.5 g), ferrous sulphate (0.01 g), $K_2Cr_2O_7$ (0.1 g), and agar (18 g) were dissolved in 0.5 liters natural sea water and 0.5 liters tap water. The pH of the solution was adjusted to 7.2 before sterilisation.

Optimisation of the Medium for Secondary Metabolite Production

The crude extract obtained by incubating *Actinomadura* sp. isolate M048 using our standard conditions (The medium is set to pH 7.8 before sterilisation and incubated at 28°C while stirring with 95 rpm) for the screening of marine streptomycetes, showed neither any activity in the biological nor any interesting zones in the chemical screening. Among various media compositions and growth conditions, the SM medium at conditions I and III afforded the highest activity against the bacteria *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü 57, SV), *Escherichia coli*, and *Staphylococcus aureus*, the fungi *Mucor miehei* and *Candida albicans*, and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* (Table 5).

Fermentation of *Actinomadura* sp. Isolate M048 and Isolation of Chandrananimycins

Starter cultures of the marine *Actinomadura* sp. isolate M048 were grown on M_2^+ agar plates with 50% sea water. After incubation for 72 hours at 28°C the strain formed a white aerial mycelium and yellow vegetative colonies with a yellow agar colouration. Well-grown agar plates were used to inoculate 100×1-liter-Erlenmeyer flasks each containing 250 ml of SM medium adjusted to pH 7.8 (condition III, Table 4) before sterilisation. The culture was grown for 4 days at 28°C while shaking at 95 rpm, which resulted in a dark brown fermentation broth.

The entire culture broth was mixed with *ca.* 1 kg diatom earth, pressed through a pressure filter and both filtrate and residue were extracted repeatedly with ethyl acetate. The combined extracts were evaporated at 30°C under vacuum to yield 11 g of a brown residue. The crude extract was triturated with MeOH (300 ml) and the insoluble solid was separated by filtering through a sintered glass funnel to get the solution A and the solid B. Solution A was evaporated and the residue subjected to silica gel column chromatography (3×60 cm, 270 g silica gel) with a stepwise cyclohexane/ethyl acetate gradient [cyclohexane (1 liter), cyclohexane with 5% (1.5 liters), 10% (1 liter), 20% (1 liter), 50% (1 liter), and 70% ethyl acetate (0.5 liters)]. Eluates were monitored by TLC and pooled into fractions I (240 mg), II (440 mg), III (140 mg) , IV (580 mg), and V (350 mg). Further purification of the fractions (Fig. 1) delivered iodinin (**1**, Rf=0.95), 1,6-

Table 5. Results of the optimisation of *Actinomadura* sp. isolate M048 (pH values were adjusted with dilute NaOH before sterilisation).

Medium	Growth Condition	Activity								
		BS	SV	EC	SA	MM	CA	CV	CS	SS
M_2^+ medium	I	0	0	0	11	0	0	11	11	0
	II	0	0	0	0	0	0	13	12	0
	III	0	0	0	11	0	0	12	0	0
SM medium	I	11	15	0	21	22	15	18	12	11
	II	0	0	0	15	0	0	17	13	0
	III	11	22	0	21	20	0	18	20	0
ME medium	I	0	19	0	15	15	0	14	13	0
	II	0	12	0	15	14	0	0	13	0
	III	0	0	0	15	12	0	0	11	0
LB medium	I	0	0	0	13	0	0	0	0	0
	II	0	13	0	15	0	0	12	0	11
	III	0	0	0	11	0	0	0	0	0
FF medium	I	12	11	0	15	12	0	0	11	0
	II	0	15	0	19	0	0	16	13	14
	III	12	15	0	15	12	0	11	11	0

I = start pH 6.5, growth temp. 28 °C, II = start pH 6.5, growth temp. 35 °C, III = start pH 7.8, growth temp. 28 °C; EC = *Escherichia coli*, BS = *Bacillus subtilis*, SA = *Staphylococcus aureus*, MM = *Mucor miehei*, CA = *Candida albicans*, CV = *Chlorella vulgaris*, CS = *Chlorella sorokiniana*, SS = *Scenedesmus suspicatus*

phenazinediol (**2**, Rf=0.42), questiomycin A (**3a**, Rf=0.46), *N*-acetylquestiomycin A (**3b**, Rf=0.63) and genistein and three new antibiotics chandrananimycin A (**3c**), B (**3d**) and C (**4**). Solid B yielded solely **1** (110 mg, Fig. 1).

Acknowledgements

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