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New Aminophenoxazinones from a Marine *Halomonas* sp.: Fermentation, Structure Elucidation, and Biological Activity

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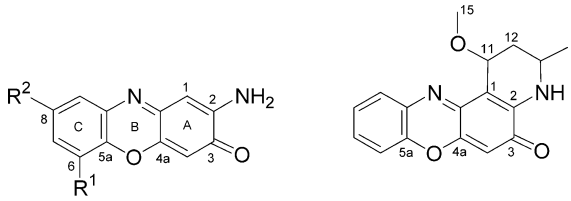
Abstract The addition of anthranilic acid to the culture medium of the marine derived *Halomonas* sp. strain GWS-BW-H8hM completely altered the secondary metabolite pattern relative to the standard conditions. The red-orange color of the culture filtrate extract was the result of the production of 2-aminophenoxazin-3-one (**1**), chandrananimycin C (**5**) and three new derivatives of **1** with a previously unknown substitution pattern: 2-amino-, 2-amino-8-benzoyl-, and 2-amino-8-(4-hydroxybenzoyl)-6-hydroxyphenoxazin-3-one (**2**∼**4**). The compounds were determined to have antibacterial and cytotoxic activities; a mode of action other than DNA intercalation is discussed.

Keywords aminophenoxazones, structure elucidation, marine bacteria, *Halomonas*, antibiotics

Introduction

The strain GWS-BW-H8hM belongs to the genus *Halomonas* and hence to the γ-proteobacteria. It was isolated from a water sample collected in the East Frisian Wadden Sea and its identification is described in related studies [1]. The strain was found to produce 3-(4′-hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (HPPD-1), 3,4-di-(4′-hydroxy-phenyl)pyrrole-2,5-dicarboxylic acid (HPPD-2) and the literature-known indole derivatives 3-(hydroxyacetyl)-indole, indole-3-carboxylic acid, indole-3-carboxaldehyde, and indole-3-acetic acid. Both HPPD-1 and HPPD-2 show potent anti-tumor-promoting activities. Their production could be improved by optimization of the fermentation conditions [1].

Addition of anthranilic acid to the medium caused a black color of both cells and culture broth. Besides non-soluble pigments-probably melanin- and known colorless diketopiperazines (**6**∼**10**) orange-colored metabolites were produced. The latter were soluble in organic solvents and showed a typical UV absorption at 430 nm. From the colored fraction, five 2-aminophenoxazin-3-one derivatives (**1**∼**5**, Fig. 1) were isolated. The new metabolites **2**, **3** and **4** exhibit a previously unknown substitution pattern of the core structure (**1**). The present work focuses on the production, isolation and structure elucidation of the new aminophenoxazinones and their biological activities.



1: $R^1 = R^2 = H$
2: $R^1 = OH$, $R^2 = H$
3: $R^1 = OH$, $R^2 =$ Benzoyl
4: $R^1 = OH$, $R^2 =$ *p*-OH-Benzoyl

5

Fig. 1 Structural formulae of the isolated aminophenoxazinones **1**∼**5**.

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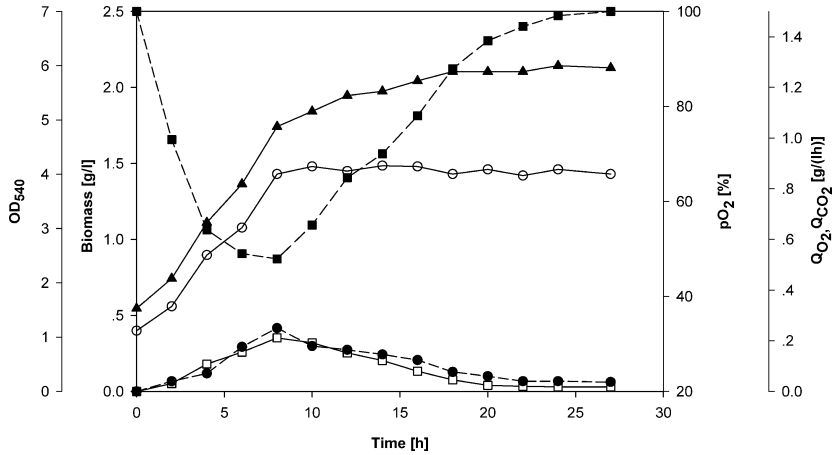

Fig. 2 Batch cultivation of *Halomonas* sp. strain GWS-BW-H8hM.

Data on growth (○), on oxygen partial pressure and gas balance. OD_{540} (▲), optical density at 540 nm; Q_{O_2} (□), oxygen uptake rate; Q_{CO_2} (●), carbon dioxide formation rate; pO_2 (■), oxygen partial pressure.

Results

Production and Isolation

Halomonas sp. strain GWS-BW-H8hM produces pigments, which cannot be obtained under standard conditions [1], when grown in artificial seawater containing peptone, yeast extract, and anthranilic acid as carbon and nitrogen sources.

A medium containing tryptone 1%, yeast extract 0.5%, glycine 0.05%, and anthranilic acid 0.05% was applied in a bioreactor cultivation. Fig. 2 presents typical data of the cultivation: The maximum for biomass of 1.6 g/liter was reached after 8 hours. During cultivation, a significant increase of OD_{540} for the cell suspension was observed. Compared with the small values of biomass, this increase is due to the pigment formation, which goes parallel to the consumption of anthranilic acid. The physiological activity, indicated by the pO_2 electrode data, the oxygen consumption rate (Q_{O_2}), and the carbon dioxide production rate (Q_{CO_2}), was connected with cell growth. From the beginning of the cultivation to 8 hours, the Q_{O_2} and Q_{CO_2}(maximum values with 0.2 g/(liter·hour) and 0.25 g/(liter·hour)) increased significantly, while the pO_2 (minimum value with 49%) decreased during this time period. After a cultivation time of 27 hours the workup of three liters of the whole broth led to 970 mg of a crude extract. The metabolites were isolated as shown in Fig. 3 by applying gel-chromatography with Sephadex LH-20, silica gel chromatography and preparative HPLC. The isolation resulted in 6.0 mg/liter of **1**, 1.0 mg/liter of **2**, 0.7 mg/liter of **3**, 0.5 mg/liter of **4** and 1.5 mg/liter of **5**. The diketopiperazines **6**~**10** were found in yields between 0.3 and 3.0 mg/liter.

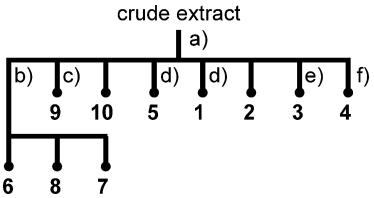

Fig. 3 Isolation scheme of the secondary metabolites from *Halomonas* sp. strain GWS-BW-H8hM.

a) Sephadex® LH-20, methanol; b) HPLC, Nucleodur RP-18, program 1; c) Sephadex® LH-20, acetone; d) silica gel, chloroform/methanol 95 : 5; e) HPLC, Nucleodur RP-18, program 2; f) silica gel, cyclohexane/ethyl acetate/methanol, gradient 5 : 10 : 0 to 5 : 10 : 1.

Structure Elucidation

2-Aminophenoxazin-3-one (**1**) was identified by its characteristic UV maxima at 237, 412 and 431 nm. The EI-MS spectrum exhibited the molecular mass at m/z=212. Comparison of the ^{1}H and ^{13}C NMR spectra with published data [2, 3] proved the identity of the compound. The assignment of the ^{13}C NMR signals (Table 1) to the carbon atoms was reinvestigated and corrected. The carbonyl group (C-3, δ_C=180.1) and the carbon atom in beta-position (C-1, δ_C=98.4) were found in the typical spectral region. HSQC and HMBC correlations were used to determine the correct assignment of the other carbon atoms. The quaternary carbons C-5a (δ_C=147.3) and C-9a (δ_C=148.2) were not distinguishable by 2D-NMR. Chemical shift calculations [4] support a more down field value for C-9a.

Compound **2** could be recognized as an aminophenoxazinone derivative by its characteristic UV spectrum, while an additional absorption maximum at

278 nm indicated small structural modifications of the chromophore. The molecular formula $C_{12}H_8N_2O_3$ was determined by HRESI mass spectrometry (m/z=229 $[M+H]^+$) and suggests an additional hydroxy group compared with **1**. The ^{1}H NMR spectrum (Table 2) shows three aromatic protons in ABC-position (J=8.0 and 1.5 Hz). Thus, the hydroxy group has to be either in position 6 or 9 of **1**. These structures could be distinguished by a detailed analysis of the ^{13}C NMR chemical shifts. A hydroxy group at C-9 would lead to a significant down field shift of C-5a and C-9 due to the electron withdrawing effects of O-5 and O-9, while C-9a would show a considerable up field shift because of the +M-effect of the oxygen atoms. This was not observed; instead the data of C-9a (δ_C=131.2), C-5a (δ_C=134.8), and C-6 (δ_C=146.0) (Table 1) proof the hydroxylation at C-6. The relative up field shifts of both C-5a and C-6 are explained by the *ortho* position of the oxygen atoms, so that their −I and +M-effects partially cancel out each other. In conclusion, compound **2** could be identified as 2-amino-6-hydroxyphenoxazin-3-one.

Table 1 ^{13}C NMR data of aminophenoxazinones **1**∼**3** (DMSO-d_6, 150 MHz; Compound **1**: 75 MHz)

Atom	**1**	**2**	**3**
1	98.4	98.4	98.2
2	148.8	148.7[a]	148.8[a]
3	180.1	180.0	180.2
4	103.3	103.0	103.9
4a	147.3	147.3[a]	147.6[a]
5a	141.9	134.8	134.1
6	115.8	146.0	145.3
7	128.7	115.5	114.9
8	125.2	124.5	132.7[b]
9	127.9	117.4	120.6
9a	133.7	131.2	134.0[b]
10a	148.2	147.8[a]	148.4[a]
1′	—	—	194.4
2′	—	—	137.1
3′/7′	—	—	129.3
4′/6′	—	—	128.5
5′	—	—	132.4

[a,b] Assignments bearing the same superscript may be interchangeable.

With respect to compound **2**, the UV spectrum of **3** changed in the area of 300 nm and pointed to an additional substituent at the chromophore. HRESI mass spectrometry (m/z=333 $[M+H]^+$) established the molecular formula $C_{19}H_{12}N_2O_4$. The ^{13}C NMR spectrum is similar to that of **2** but it includes the signals of an additional carbonyl group (δ_C=194.4) and six aromatic carbons of a phenyl residue (Table 1). The ^{1}H NMR spectrum shows only two aromatic protons of ring C ($^4J_{HH}$=2.0 Hz), giving evidence for their *meta*-position (Table 2). These protons as well as those of the phenyl residue show HMBC correlations to the carbonyl group at δ_C=194.4. Consequently, compound **3** can be identified as 2-amino-8-benzoyl-6-hydroxyphenoxazin-3-one.

The ^{1}H NMR spectrum of compound **4** is similar that of **3** (Table 2). However, instead of a phenyl group the spectrum exhibits two 2H doublets with a coupling constant of $^3J_{HH}$=8.5 Hz. Their chemical shifts (δ_H=6.81 and 7.65) indicate a *para*-disubstituted aromatic ring with an oxygen in *para*-position. HRESI mass spectrometry (m/z=349 $[M+H]^+$) confirmed the molecular formula $C_{19}H_{12}N_2O_5$. Thus compound **4** differs from **3** by the presence of a hydroxy group and could be assigned as 2-amino-8-(4-

Table 2 ^{1}H NMR data of aminophenoxazinones **1**∼**4** (DMSO-d_6, 600 MHz)

Atom	**1**	**2**	**3**	**4**
1	6.34, s	6.31, s	6.36, s	6.34, s
4	6.37, s	6.37, s	6.41, s	6.37, s
6	7.48, dd (8.0, 1.5)	—	—	—
7	7.45, td (8.0, 1.5)	6.99, dd (8.0, 1.5)	7.38, d (2.0)	7.30[a], m
8	7.38, td (8.0, 1.5)	7.15, t (8.0)	-	—
9	7.69, dd (8.0, 1.5)	7.10, dd (8.0, 1.5)	7.42, d (2.0)	7.23[a], d (2.0)
NH_2	6.76, br s	6.69, br s	6.88, br s	6.73, br s
3′/7′	—	—	7.76, d (7.5)	7.74, d (9.0)
4′/6′	—	—	7.59, t (7.5)	6.85, d (9.0)
5′	—	—	7.69, t (7.5)	—

[a] assignments may be interchangeable.

hydroxybenzoyl)-6-hydroxyphenoxazin-3-one.

The compound of fraction 4 (EI-MS: m/z=296) was identified as chandrananimycin C (**5**) by comparison of the NMR data given in the literature [3].

In the more lipophilic fractions 1～3 of the crude extract (Fig. 3), five proline-containing diketopiperazines were found as metabolites of *Halomonas* sp. The structures of cyclo(prolylvalyl) (**6**), cyclo(leucylprolyl) (**7**), cyclo(isoleucylprolyl) (**8**), cyclo(phenylalanylprolyl) (**9**), and cyclo(prolyltyrosyl) (**10**) (Fig. 4) were elucidated by comparison of their ^{1}H NMR and EI-MS spectra with published data [5, 6]. Diketopiperazines are produced by condensation of two amino acids and are widespread as metabolites of various bacteria and fungi. Natural diketopiperazines show a remarkably preference for proline as a building block [7]. They consist mostly of two L-amino acids, but DL-, LD-, and DD-isomers can be generated by non-enzymatic epimerization, possibly accelerated by neighboring groups [8].

Biological Activity

The aminophenoxazinones **1**～**5** were tested against different bacteria and *Candida albicans* in a plate diffusion assay (Table 3). The strongest growth inhibition of Gram-positive bacteria was observed for compound **2**. The core structure itself (compound **1**) as well as the more lipophilic derivative **3** had weaker activities. The experimental compounds were significantly less potent than the reference compound, actinomycin D. No antimicrobial activity was observed for compounds **4** and **5**; the latter result is in contrast to previous reports [3]. Aminophenoxazinone **1** was the only metabolite that exhibited activity against *C. albicans.*

Compounds **1**, **2** and **3** are moderately cytotoxic against different tumor cell lines (Table 4). The effect of actinomycin D is about 2 to 3 orders of magnitude higher. Compound **1** with the aminophenoxazinone core structure exhibited a stronger cytotoxicity than the derivatives of **1**. The results of a cell cycle analysis of **1** are given in Table 5.

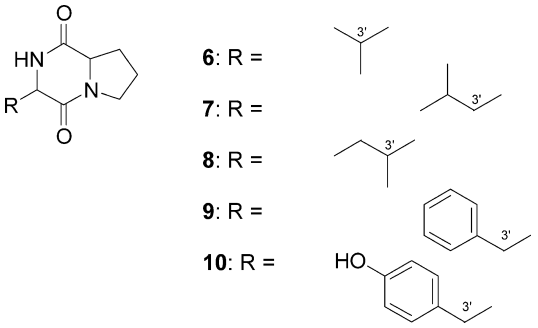

Fig. 4 Structural formulae of the isolated diketopiperazines **6**～**10**.

Table 3 Antimicrobial activity of the aminophenoxazinones **1**～**3** and actinomycin D in a plate diffusion assay

Test organism	*E. coli*	*B. subtilis*	*S. aureus*	*C. albicans*
1	0	20	15	25
2	0	25	16	0
3	0	16	10	0
Actinomycin D	16	35	25	0

Diameter of the inhibition zones in mm, 50 μg of compound on 6 mm filter discs.

Table 4 Cytotoxicities of the aminophenoxazinones **1**～**3** against different tumor cell lines: HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF7 (breast adenocarcinoma) (GI_{50} and TGI values in μg/ml)

Cell line	HM02		HepG2		MCF7	
	GI_{50}	TGI	G_{I50}	TGI	GI_{50}	TGI
1	0.95	2.2	1.4	5.6	0.13	0.42
2	1.6	2.6	4.3	>10	1.6	2.7
3	1.4	2.6	3.2	10	2.0	3.2
Actinomycin D	0.002	0.008	0.0015	0.0065	0.0024	0.011

GI_{50} is the concentration at which half of the cells were inhibited in their growth, and TGI is the concentration at which a total inhibition of cell growth was observed.

Table 5 Cell cycle investigation with HM02 cells (% cells in the specified cell phase±standard deviation).

Cell phase	Sub G1 (apoptose)	G0/G1	S	G2/M
Control	3.6±1.3	54.5±3.5	19.7±0.8	21.5±5
1	20±11	34±5*	21.5±6	22.7±0.4
Actinomycin D	6.0±3.4	54.0±6.5	10.0±1.6*	29.2±8.5

* $p<0.05$ *versus* control (*t*-test)

Discussion

The addition of anthranilic acid to the culture medium of *Halomonas* sp. (strain GWS-BW-H8hM) changes the metabolite pattern completely and leads to the production of five aminophenoxazinones. Thus, the manipulation of this marine strain is a good example for the OSMAC (one strain - many compounds) approach [9] to the production of novel secondary metabolites. The core structure 2-aminophenoxazin-3-one (**1**) is the main product and known to originate biosynthetically from an oxidative coupling of two units of anthranilic acid [10]. **1** can undergo a regioselective hydroxylation at C-6 to form **2**. Furthermore, an acylation of **2** with a benzoyl or 4-hydroxybenzoyl residue at C-8 can be assumed. These groups may be derived from phenylalanine and tyrosine, respectively.

Compound **2** is the first-known aminophenoxazinone derivative substituted at C-6 only. The 6,8-disubstituted compounds **3** and **4** as well represent a previously unknown substitution pattern. In all other known aminophenoxazinones, a residue in position 6 is always accompanied with a second substitution at position 9. This pattern is known for instance from the actinomycins, cytotoxic chromopeptides with an aminophenoxazinone chromophore [11].

Unexpectedly, 2-amino-6-hydroxyphenoxazin-3-one (**2**) exhibited a higher antibacterial activity than the core structure **1**. Assuming a mode of action based on intercalation with the DNA double helix, any modifications at the chromophore should weaken the interaction, and consequently the antibacterial activity. The opposite effect could be the result of a different mode-of-action, which is supported by cell cycle investigations with HM02 cells (Table 5). Actinomycin D reduces significantly the ratio of cells in the S-phase, as expected for an intercalating DNA inhibitor [12]. In contrast, 2-aminophenoxazin-3-one (**1**) reduces the ratio of cells in the resting phases (G0/G1) and forces the cells directly to apoptosis. These experiments demonstrate that **1** represses the cells' transition from the resting phase to the synthesis phase and hence induces programmed cell death.

Experimental

General

NMR spectra were recorded on Varian Inova 600 and Unity 300 spectrometers. Chemical shifts are expressed in δ values using the solvent as internal standard (DMSO-d_6: δ_H 2.50, δ_C 39.5; $CDCl_3$: δ_H 7.25, δ_C 77.0). Coupling constants are given in Hz. EI mass spectra were recorded on Finnigan MAT 95 (70 eV); relative intensities in parenthesis refer to the highest peak of the spectrum. ESI-MS spectra were obtained on Finnigan LC-Q and HRESI-MS spectra on Bruker Apex-Q III (field strength 7 Tesla). Infrared spectra were recorded on a Perkin-Elmer model 1600 as KBr pellets. UV spectra were obtained in methanol on a Varian Cary 3E spectrometer.

Fermentation and Growth

Initial growth of *Halomonas* sp. GWS-BW-H8hM was achieved on Marine Broth 2216 (Difco, Germany). Slants were stored at 4°C and transferred on monthly intervals. Liquid cultures (1000 ml and 2000 ml Erlenmeyer flasks, using 250 ml and 500 ml broth volumes, respectively) were incubated at 27°C in a shake-incubator rotating at 100 rpm for 72 hours. The artificial seawater medium contained per liter: Fe citrate 0.1 g, NaCl 19.45 g, $MgCl_2 \cdot 6H_2O$ 8.8 g, Na_2SO_4 3.24 g, $CaCl_2 \cdot 2H_2O$ 2.38 g, Na_2HPO_4 0.01 g, SiO_2 0.015 g, trace elements [13] 1 ml, stock solution for artificial seawater 10 ml. The optimized medium contained the following supplements: tryptone 10 g, yeast extract 5 g, glycine 0.5 g, and anthranilic acid 0.5 g in 1 liter. For a batch cultivation in artificial seawater medium a 30-liter bioreactor (Biostat P, Braun Diessel, Melsungen, Germany) was used with the following conditions: 20 liters working volume, inoculation with 10% (v/v) of a 24-hour

preculture; temperature 27°C, stirring at 250 rpm, aeration rate 0.1 v/vm, pH adjusted to 7.5. The bioreactor was equipped with a six-bladed disc turbine, and the physiological activity was followed by the use of a pO_2-electrode, and by oxygen and carbon dioxide gas analyzers (Oxygor and Unor, Maihak, Hamburg, Germany). For biomass measurements, 20 ml of cell culture were centrifuged for 30 minutes at 13,000 rpm and at 4°C. After drying at 105°C, the biomass was determined gravimetrically.

Isolation

The pH value of three liters of whole broth was adjusted to pH 3.0 (HCl). After extraction with six liters ethyl acetate, the solvent was evaporated at 240 mbar and by brief freeze-drying. The products were purified as shown in Fig. 3. Silica gel 60 (0.2～0.05 mm, Macherey-Nagel) and Sephadex® LH-20 (Sigma Aldrich) were used for column chromatography. Preparative high performance liquid chromatography: Jasco PU-1587 pump; Jasco UV-1575 UV-detector; Rheodyne manual injection valve; software Jasco Borwin™ Version 1.50 with Jasco HSS-2000 Version 3.5.2; column Jasco Nucleosil 100 C18, 5 μm, 250×8 mm, flow rate 2.6 ml/minute; solvent system: water (A)/acetonitrile (B), both with 0.1% TFA; program 1: 13 minutes at 17% B, in 1.5 minutes to 90% B, 5 minutes at 90% B, in 1.5 minutes to 17% B, 2 minutes at 17% B, UV-detection at 215 nm; program 2: 7 minutes at 22% B, in 1 minute to 44% B, 10 minutes at 44% B, in 1 minute to 88% B, 5 minutes at 88% B, in 2 minutes to 22% B, 2 minutes at 22% B, UV-detection at 254 nm.

Biological Tests

For plate diffusion tests 50 μg of the desired compound were solved in methanol and dropped on paper disks (diameter 6 mm, thickness 0.5 mm). These were dried under sterile conditions and put on agar plates inoculated with the test organism (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*). The plates were cultivated at 37°C (bacteria) or 25°C (yeast) for 24 hours.

The cytotoxic activity was determined according to the NCI (USA) protocol [14] with the tumor cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF7 (breast adenocarcinoma). The cells were cultivated in 96-well micro titer plates with medium RPMI 1640 with 10% fetal bovine serum. After 24 hours the test compounds were added as DMSO solutions (c=0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 10 μg/ml) and the cells cultivated for additional 48 hours. The cell count was surveyed by protein determination with sulforhodamin. From the resulting concentration-activity curves, the GI_{50} (concentration at which half of the cells were inhibited in their growth) and TGI values (concentration at which a total inhibition of cell growth was observed) were obtained.

Cell cycle distributions were determined with HM02 cells that were incubated for 24 hours in 12-well plates with RPMI 1640 with 10% fetal bovine serum. After addition of the test compound as DMSO solution (10 μg/ml) the cells were cultivated for another 24 hours. The supernatant was taken off and the cells washed with 300 μl ice cold PBS solution. The PBS solution was transferred in 4.4 ml 100% ethanol (ice cooled). The adherend cells were incubated with 300 μl trypsin solution (37°C). After 2 minutes the supernatant was added (1 ml) and the cell suspension (1.3 ml) repeatedly soaked up with a pipette before it was transferred in the ethanol solution. The cell suspension was stored for 30 minutes on ice and afterwards centrifuged at 3000 rpm. The supernatant was discarded, the pellet resuspended in 0.5 ml PBS solution with 1% BSA and centrifuged at 4500 rpm for 5 minutes. The supernatant was taken off and the pellet solved in 100 μl staining solution (propidium bromide 150 μg/ml, 4 mM Na citrate pH 7.0, Triton C-100 1%, BSA 1%). After 15 minutes incubation at room temperature in the dark, 100 μl RNAse solution (10 mg/ml Ribonuclease A in Tris/HCl buffer, pH 7.5) were added. Thirty minutes later the cell suspension was counted by FACSscan.

2-Aminophenoxazin-3-one (1)

Red solid; Rf 0.54 (CH_3Cl/MeOH, 9 : 1); EI-MS (70 eV) m/z (%) 212 (100) [M, $C_{12}H_8N_2O_2]^+$, 185 (35); UV λ_{max}^{MeOH} nm (log ε) 237 (4.34), 421 (4.26), 431 (4.26); IR $\tilde{\nu}_{max}$ (KBr) cm^{-1} 3407, 1580, 1472, 1420, 1278, 1176, 1106, 1054; ^{1}H NMR (600 MHz, DMSO-d_6) see Table 2; ^{13}C NMR (75.5 MHz, DMSO-d_6) see Table 1.

2-Amino-6-hydroxyphenoxazin-3-one (2)

Red solid; Rf 0.43 (CH_3Cl/MeOH, 9 : 1); EI-MS (70 eV) m/z (%) 228 (100) [M, $C_{12}H_8N_2O_3]^+$, 201 (40); HRESI-MS calcd m/z 229.06077 $[M+H]^+$, found m/z 229.06079; UV λ_{max}^{MeOH} nm (log ε) 236 (3.93), 277 (3.75), 430 (3.72);IR $\tilde{\nu}_{max}$ (KBr) cm^{-1} 3431, 1643 (sh), 1589, 1506, 1460, 1382, 1277, 1212, 1085, 1027; ^{1}H NMR (600 MHz, DMSO-d_6) see Table 2; ^{13}C NMR (150 MHz, DMSO-d_6) see Table 1.

2-Amino-8-benzoyl-6-hydroxyphenoxazin-3-one (3)

Red solid; Rf 0.49 (CH_3Cl/MeOH, 9 : 1); ESI-MS m/z pos. 333 $[M+H, C_{19}H_{13}N_2O_4]^+$, 687 $[2M+Na]^+$; neg. 331 $[M-H]^-$, 663 $[2M-H]^-$; HRESI-MS calcd m/z 333.086983 $[M+H]^+$, found m/z 333.086901; UV λ_{max}^{MeOH} nm (log ε) 257 (3.81), 309 (3.59), 432 (3.46); IR $\tilde{\nu}_{max}$ (KBr) cm^{-1} 3435, 2943, 2361, 1670, 1638, 1432, 1264,

1202, 1137, 1088, 1034; ^{1}H NMR (600 MHz, DMSO-d_6) see Table 2; ^{13}C NMR (150 MHz, DMSO-d_6) see Table 1.

2-Amino-8-(4-hydroxybenzoyl)-6-hydroxyphenoxazin-3-one (4)

Red solid; Rf 0.32 (CH_3Cl/MeOH, 9 : 1); ESI-MS *m/z* pos. 349 $[M+H, C_{19}H_{13}N_2O_5]^+$, 719 $[2M+Na]^+$; neg. 347 $[M-H]^-$, 695 $[2M-H]^-$; HRESI-MS calcd *m/z* 349.08190 $[M+H]^+$, found *m/z* 349.08176; UV λ_{max}^{MeOH} nm (log ε) 266 (3.54), 300 (3.52), 428 (3.44), 434 (3.45); IR $\tilde{\nu}_{max}$ (KBr) cm^{-1} 3432, 1618, 1584, 1398, 1265, 1176, 1109, 1029; ^{1}H NMR (600 MHz, DMSO-d_6) see Table 2.

Chandrananimycin C (5)

Red solid; Rf 0.78 (CH_3Cl/MeOH, 9 : 1); EI-MS (70 eV) *m/z* (%) 296 (42) $[M, C_{17}H_{16}N_2O_3]^+$, 281 (20), 266 (100), 265 (56), 264 (50), 263 (42), 250 (27), 249 (100), 238 (20), 221 (36); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36 (m, 1H, 12-H_a), 1.37 (d, J=6.5 Hz, 3H, 14-H_3), 2.22 (m, 1H, 12-H_b), 3.53 (s, 3H, OMe), 3.73 (m, 1H, 13-H), 5.07 (t, J=3.0 Hz, 1H, 11-H), 5.88 (br s, 1H, NH), 6.35 (s, 1H, 4-H), 7.35 (m, 3H, 6-H, 7-H, 8-H), 7.76 (dt, J=7.0, 1.5 Hz, 1H, 9-H).

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References

1. Wang L, Große T, Stevens H, Brinkhoff T, Simon M, Liang L, Bitzer J, Bach G, Zeeck A, Tokuda H, Lang S. Bioactive hydroxyphenylpyrrole-dicarboxylic acids from a new marine *Halomonas* sp.: Production and structure elucidation. Appl Microbiol Biotechnol In press
2. Hasegawa K, Ueno Y. The carbon-13 NMR spectra and electronic structure of 3H-phenoxazin-3-ones. Bull Chem Soc Jpn 58: 2832–2839 (1985)
3. Maskey RP, Li FC, Qin S, Fiebig HH, Laatsch H. Chandrananimycins A∼C: Production of novel anticancer antibiotics from a marine *Actinomadura* sp. isolate M048 by variation of medium composition and growth conditions. J Antibiot 56: 622–629 (2003)
4. ACD/CNMR Predictor, Version 6.12. Advanced Chemistry Development Inc., Toronto (2002)
5. Fdhila F, Vazquez V, Sánchez JL, Riguera R. DD-Diketopiperazines: Antibiotics active against *Vibrio anguillarum* isolated from marine bacteria associated with cultures of *Pecten maximus*. J Nat Prod 66: 1299–1301 (2003)
6. De-hai L, Quian-qun G, Wei-ming Z, Hong-bing L, Yu-chun F, Tian-jiao Z. Antitumor components from marine actinomycete 11014. I. Cyclic dipeptides. Chin J Antibiot 30: 449–452 (2005)
7. Bull SD, Davies SG, Parkin RM, Sánchez-Sancho F. The biosynthetic origin of diketopiperazines derived from D-proline. J Chem Soc, Perkin Trans 1 2313–2320 (1998)
8. Smith GG, Evans RC, Baum R. Neighboring residue effects: Evidence for intramolecular assistance to racemization or epimerization of dipeptide residues. J Am Chem Soc 108: 7327–7332 (1986)
9. Bode HB, Bethe B, Höfs R, Zeeck A. Big effects from small changes: Possible ways to explore nature's chemical diversity. ChemBioChem 3: 619–627 (2002)
10. Barry CE, Nayar PG, Begley TP. Phenoxazinone synthase: Mechanism for the formation of the phenoxazinone chromophore of actinomycin. Biochemistry 28: 6323–6333 (1989)
11. Mauger AB, Lackner H. The Actinomycins. *In* Anticancer agents from natural products. *Eds.* Kingston DGI, Cragg GM & Newman DJ, pp. 281–298, CRC Press, Boca Raton (2005)
12. Wu MH, Yung BY. Cell cycle phase-dependent cytotoxicity of actinomycin D in HeLa cells. Eur J Pharmacol 270: 203–212 (1994)
13. Hoagland H. Quantitative analysis of responses from lateraline nerves of fishes. J Gen Physiol 16: 715–732 (1933)
14. Grever MR, Schepartz SA, Chabner BA. The National Cancer Institute: Cancer drug discovery and development program. Semin Oncol 19: 622–638 (1992)