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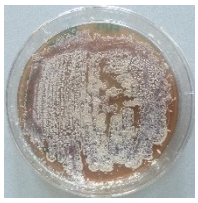

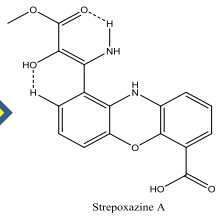

Strepoxazine A

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Strepoxazine A, a new cyctotoxic phenoxazin from the marine sponge-derived bacterium *Streptomyces* sp. SBT345

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ABSTRACT

One new phenoxazin analogue, strepoxazine A (1), along with two known phenazine compounds phencomycin (2) and tubermycin B (3) were isolated from the solid culture of *Streptomyces* sp. SBT345 which had previously been recovered from the Mediterranean sponge *Agelas oroides*. The structures of compound 1, 2, and 3 were determined by spectroscopic analysis including 1D and 2D NMR, HR-ESI-MS experiments as well as comparison to literatures. We investigated the apoptotic effect of the three compounds on the human promyelocytic leukemia cells HL-60 and human breast adenocarcinoma cells MCF-7. Only strepoxazine A (1) showed cytotoxicity against HL-60 cells with IC_{50} at 16 µg/ml. These results demonstrate that sponge-associated actinomycetes are rich sources for natural products with new pharmacological activities and relevance to drug discovery.

The quest for novel biological structures is a challenge in natural product-directed drug discovery. Marine environment has been offering a broad and valuable resource for human's long existence and development. Its biological richness and diversity attracted researchers to seek for unique and novel molecules from the previously underexplored sources, such as the deep sea sediments, marine algae, marine invertebrates, and their associated microbes. Marine sponge-associated microorganisms have exhibited a great potential in producing natural products with diverse medicinal and pharmaceutical properties [1-5]. In particular, sponge-associated actinomycetes made good records in providing a multitude of novel secondary metabolites [6, 7] with diverse biological activities, such as antimicrobial [8, 9], anti-parasitic [10, 11], immunomodulatory [12], antichlamydial [13], anti-oxidant [7], and anticancer [14, 15] activities. Herein, we report on the structures and anti-cancer activities of one new phenoxazin analogue strepoxazine A (**1**), and two known phenazine analogues phencomycin (**2**) and tubermycin B (**3**) obtained from the solid extract of sponge-associated *Streptomyces* sp. SBT345.

Streptomyces sp. SBT345, recovered from the Mediterranean sponge *Agelas oroides*, was plated out on ISP2 agar medium plate (120×120 mm, a total of 150 plates) and incubated at 30 °C for 5 days. Colonies and agar were cut into small pieces and macerated with ethyl acetate. The ethyl acetate extract (630 mg) was fractionated on a Sephadex LH20 (32-64 µm, 100 x 10 mm, Merck) column eluting with H_2O/MeOH (90:10%) to MeOH (100%), to yield 6 fractions. Fraction 5 was subjected to C_{18} reversed-phase chromatography and led to the isolation of compounds **1-3**.

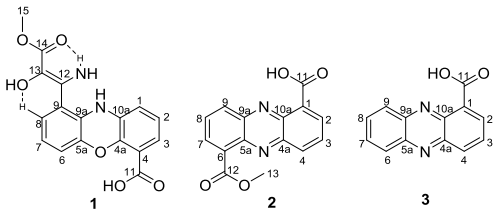

Figure 1. Structures of strepoxazine A (**1**), phencomycin (**2**), and tubermycin B (**3**)

Compound **1** was obtained as a yellowish powder from fraction 2 and the molecular formula was established as $C_{17}H_{14}N_2O_6$ by ESI-HR-MS (found at *m/z* 341.0766 $[M - H]^-$, cald. 341.0774; and *m/z* 365.0757 $[M + Na]^+$, cald. 365.0750) requiring 11 degrees of unsaturation. The ^1H NMR spectrum exhibited the resonances for

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six sp^2 aromatic proton signals at δ_H 7.38 (1H, brs, H-1), 7.01 (1H, t, J = 7.9, 15.7 Hz, H-2), 7.93 (1H, d, J = 7.9 Hz, H-3), 7.19 (1H, d, J = 7.7 Hz, H-6), 7.24 (1H, t, J = 7.7, 15.4 Hz, H-7), 7.43 (1H, brs, H-8) ppm, of which two independent aromatic systems were observed based on the analysis of COSY spectrum. The HMBC cross-peaks from the aromatic protons of H-2 to δ_C 121.5 (1C, C-10a) ppm, H-3 to δ_C 144.2 (1C, C-4a) ppm, H-7 to δ_C 142.5 (1C, C-5a) ppm, and H-6 to δ_C 125.0 (1C, C-9a) ppm led to the assignment of a phenoxazin nucleus [16], in which the carbon resonance of C-4a, C-5a, C-9a and C-10a were consistent with the other analogues in the literature exemplified by Venezueline C and Venezueline D [17]. Additional NMR data conducted to assign one carboxylic acid group to C-4 by correlation observed between H-3 and a carboxylic carbon at δ_C 173.3 (1C, C-11) ppm, and one methoxyl ester by correlation observed between the methoxyl protons at δ_H 3.88 (3H, s, H-15) ppm to the other carbonyl at δ_C 168.5 (1C, C-14) ppm in the HMBC spectrum. The presence of the carboxylic acid group was further verified in ESI-HRMS/MS spectra (collision voltage of 10eV) by the loss mass of 43.9898 Da (cald. for COO) from the molecular ion mass *m/z* 341.0766 (cald. for $C_{17}H_{13}N_2O_6^-$) to fragment ion mass *m/z* 297.0867 (cald. for $C_{16}H_{13}N_2O_4^-$) (**Figure 2**). The presence of the methoxyl ester was also verified by the loss mass of 59. 0234 Da (cald. for $C_2H_3O_2$) in both negative and positive ionisation modes from the molecular ion mass *m/z* 341.0766 (cald. for $C_{17}H_{13}N_2O_6^-$) to fragment ion mass *m/z* 282.0639 (cald. for $C_{15}H_{10}N_2O_4^-$) (**Figure 2**), as well as the molecular ion mass *m/z* 365.0757 (cald. for $C_{17}H_{14}N_2O_6Na^+$) to fragment ion mass *m/z* 306.0619 (cald. for $C_{15}H_{11}N_2O_4Na^+$) (**Figure 3**) respectively. The fragment ion mass *m/z* 265.0611 (cald. for $C_{15}H_9N_2O_3^-$) in negative ionisation mode (**Figure 2**), and *m/z* 288.0515 (cald. for $C_{15}H_9N_2O_3Na^+$) in the positive ionisation mode (**Figure 3**) were deduced by losing the carboxylic acid and methoxyl groups from the molecular ion. Furthermore, *m/z* 237.0662 (cald. for $C_{14}H_9N_2O_2^-$) and *m/z* 260.0564 (cald. for $C_{14}H_9N_2O_2Na^+$) were the products of losing both carboxylic acid group and methoxyl ester side chain (**Figure 2** and **3**). The high-resolution fragment ion masses and the NMR data assigned the phenoxazine based structure, one carboxylic acid, and one methoxyl ester side chain which established a partial elementary composition as $C_{15}H_{11}NO_5$. The remaining elements C_2H_3NO were deduced as an olefinic structure that substituted by a hydroxyl and an amine group, and connects between the methoxyl ester and the aromatic carbon C-9. The enol group was deduced to connect to C-13 due to the broad proton resonance at H-8 which could be affected by the intramolecular hydrogen bond with enol proton. The enamino proton was also speculated to form the other intramolecular hydrogen bond with the ketone oxygen at C-14 to form a keto-enamine structure, which makes the whole structure more stable than the presence of single enol or enamino group [18, 19]. Additionally, the fragment ion mass *m/z* 209.0710 (cald. for $C_{13}H_9N_2O^-$) presented in negative mass spectrum was interpreted with the structure shown in **Figure 2** by the web server named as CFM-ID designed for annotation, spectrum prediction and metabolite identification from tandem mass spectra using the data from HMDB, MassBank, and Metlin databases [20]. The deprotonated imine at C-12 (**Figure 2**) further demonstrated the elucidation of enamino part.

Compound **2** was isolated as yellow solid from fraction 3 and the molecular formula was established as $C_{15}H_{10}N_2O_4$ with 11 degrees of unsaturation by ESI-HRMS (found at m/z 283.0710 [M + H]$^+$, cald. 283.0719). The ^{1}H NMR and COSY spectra revealed six sp^2 aromatic protons at δ_H 7.67 (1, d, J = 8.7 Hz, H-2), 7.88 (1H, t, J = 8.7, 15.4 Hz, H-3), 8.02 (1H, d, J = 8.7 Hz, H-4), 8.19 (1H, d, J = 8.7 Hz, H-7), 7.97 (1H, t, J = 8.7, 15.6 Hz, H-8), and 8.38 (1H, d, J = 8.7 Hz, H-9) ppm in two different aromatic rings and 1 methoxyl proton at δ_H 4.01 (3H, s, H-13) ppm. The ^{1}H-^{13}C HMBC spectrum assigned the nine aromatic carbons δ_C 125.9 (1C, C-2), 127.3 (1C, C-4), 142.0 (1C, C-4a), 139.1 (1C, C-5a), 132.1 (1C, C-6), 131.1 (1C, C-7), 132.0 (1C, C-9), 142.1 (1C, C-9a), 140.4 (1C, C-10a) ppm, and two carboxylic carbonyls δ_C 170.5 (1C, C-11), and 167.8 (1C, C-12) ppm. The carbon resonances of C-4a, C-5a, C-9a, and C-10a indicated a phenazine based structure. An exact mass search in the Database of Natural Products and comparison of the spectral data with literature [21] determined compound **2** as a known phenazine compound phencomycin which was previously isolated from *Streptomyces* sp. derived from terrestrial [21, 22] and marine sources [23].

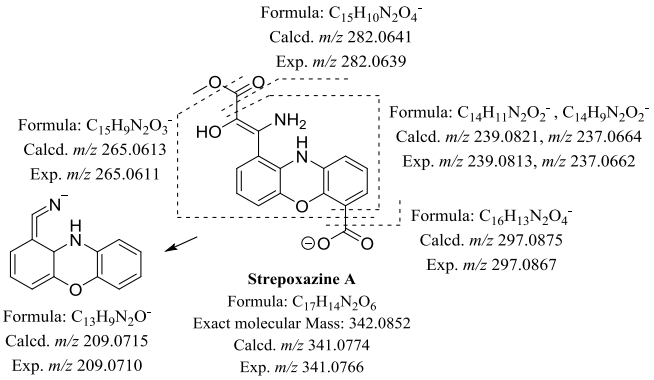

Figure 2. ESI-HRMS^2 fragmentation of strepoxazine A in negative ionization mode

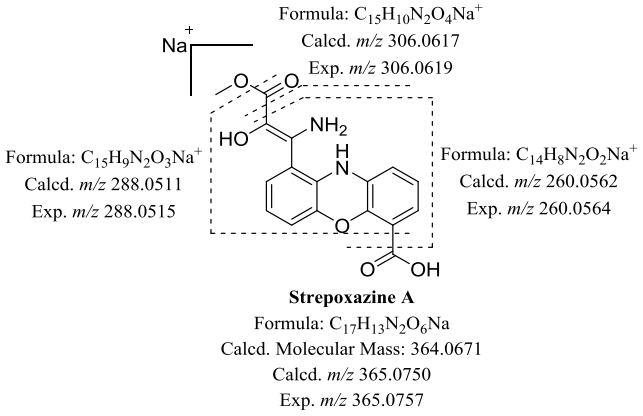

Figure 3. ESI-HRMS^2 fragmentation of strepoxazine A in positive ionization mode

Compound **3** was isolated as yellow solid from fraction 3 and the molecular formula was established as $C_{13}H_8N_2O_2$ with 10 degrees of unsaturation by ESI-HRMS (found at m/z 225.0655 [M + H]$^+$, cald. 225.0664). The ^{1}H NMR revealed seven sp^2 aromatic protons at δ_H 8.72 (1H, d, J = 8.8 Hz, H-2), 8.12 (1H, m, H-3), 8.53 (1H, d, J = 8.8 Hz, H-4), 8.37 (1H, d, J = 8.3 Hz, H-6), 8.08 (1H, m, H-7), 8.10 (1H, m, H-8), 8.34 (1H, d, J = 8.3 Hz, H-9) ppm in two different aromatic rings. The ^{1}H-^{13}C HMBC spectrum assigned eight aromatic carbons δ_C 133.3 (1C, C-2), 143.2 (1C, C-5a), 129.7 (1C, C-6), 132.2 (1C, C-7), 131.6 (1C, C-8), 129.7 (1C, C-9), 143.2 (1C, C-9a), and 140.1 (1C, C-10a) ppm. A search in Database of Natural Products and comparison of spectral data with phencomycin and literatures [24, 25] resulted in a known phenazine compound tubermycin B which was previously isolated from *Pseudomonas* sp. [26] and *Streptomyces* sp. [25].

Various phenoxazines and phenazines have been reported for their anticancer activities against a panel of tumor cell lines, including intestinal adenocarcinoma cell lines [17, 27], gastric

cancer cell lines [28-30], pancreatic cancer cell lines [31, 32], lung tumor cell lines [33-35], breast cancer cell lines [34-36], human hepatoma cell lines [17, 37, 38], multiple myeloma cell lines [39, 40], and human promyelocytic leukemia cells [41-43], etc. The antiproliferative activity of compounds **1**–**3** were evaluated against human promyelocytic leukemia cells HL-60 and human breast adenocarcinoma cells MCF-7 using Vitality Test and MTT assay. The new phenoxazine strepoxazine A (**1)** exhibited significant cytotoxic properties against HL-60 with IC_{50} at 16 µg/ml. However, the other two phenazines **2** and **3** did not display any activity.

In conclusion, strepoxazine **1** is a new phenoxazine analogue isolated from the solid culture of sponge-associated *Streptomyces* sp. SBT345. and exhibited potent apoptotic effect against human promyelocytic leukemia cells HL-60. The results presented in this paper highlight sponge-associated actinomycetes as a rich source for novel biologically active natural products.

Acknowledgments

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[16] Strepoxazine A (**1**) 1.3 mg; Yellowish powder (Rt = 17.005 min); UV (EtOH) λ_{max} 220, 290, 350 nm; IR (KBr) ν_{max} 3436, 3044, 2816, 2270, 2137, 1877, 1767, 1415, 1114 cm^{-1}; ^1H NMR (MeOD-*d*4, 600 MHz,): δ = 7.38 (1H, brs, H-1), 7.01 (1H, t, *J* = 7.9, 15.7 Hz, H-2), 7.93 (1H, d, *J* = 7.9 Hz, H-3), 7.19 (1H, d, *J* = 7.7 Hz, H-6), 7.24 (1H, t, *J* = 7.7, 15.4 Hz, H-7), 7.43 (1H, brs, H-8), 3.88 (3H, s, H-15); ^{13}C NMR (MeOD-*d*4, 150 MHz,): δ = 127.8 (CH, C-1), 120.6 (CH, C-2), 131.3 (CH, C-3), 126.1 (C, C-4), 144.2 (C, C-4a), 142.5 (C, C-5a), 119.6 (CH, C-6), 127.9 (CH, C-7), 122.9 (CH, C-8), 129.4 (C, C-9), 125.0 (C, C-9a), 121.5 (C, C-10a), 173.3 (C, C-11), 133.8 (C, C-12), 134.1 (C, C-13), 168.5 (C, C-14), 52.8 (CH3, C-15); HR-ESI-MS *m/z* 341.0766 $[M - H]^-$, $C_{17}H_{13}N_2O_6$ (calcd. 341.0774); *m/z* 365.0757 $[M + Na]^+$, $C_{17}H_{14}N_2O_6Na$ (calcd. 365.0750); Anal. Calcd. for $C_{17}H_{14}N_2O_6$: C, 59.65; H, 4.12; N, 8.18; O, 28.04.

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.