Glaciapyrroles A, B, and C, Pyrrolosesquiterpenes from a *Streptomyces* sp. Isolated from an Alaskan Marine Sediment

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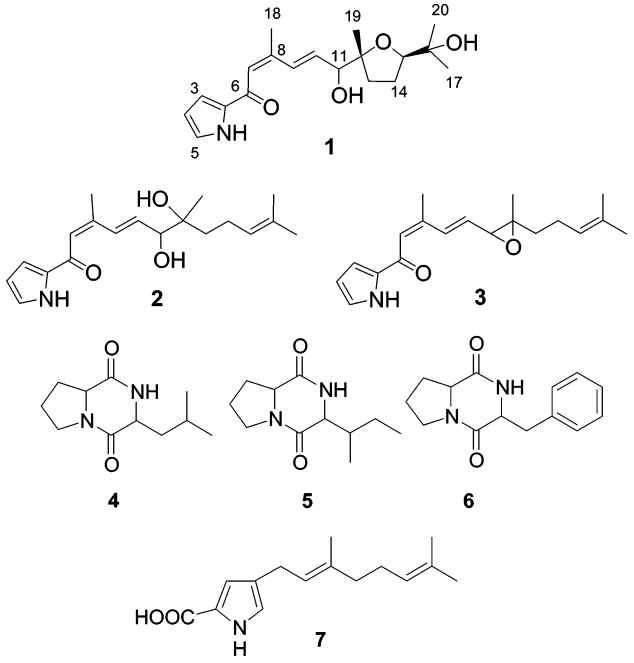
A *Streptomyces* sp. (NPS008187) isolated from a marine sediment collected in Alaska was found to produce three new pyrrolosesquiterpenes, glyciapyrroles A (**1**), B (**2**), and C (**3**), along with the known diketopiperazines cyclo(leucyl-prolyl) (**4**), cyclo(isoleucyl-prolyl) (**5**), and cyclo(phenylalanyl-prolyl) (**6**). The structures of **1**, **2**, and **3** were established using spectroscopic methods.

Marine microorganisms continue to serve as a prolific source of new natural products.[1,2] In an ongoing effort to increase the success rate for the identification of novel secondary metabolites from this source, chemical profiling of crude fermentation culture extracts has been introduced at the front end of our discovery process. One useful tool for assessing a crude extract's potential for chemical novelty is analytical HPLC with photodiode array (PDA) detection. The HPLC-PDA approach allows the UV spectra of individual peaks in the chromatogram to be acquired and matched against our in-house UV spectral library. The analysis may result in rapid conclusion of the structure elucidation through dereplication, or alternatively suggest the presence of previously unidentified natural products. Using this spectral matching approach, several peaks with unique UV spectra were identified in crude extracts derived from Nereus strain NPS008187. These extracts were targeted for follow-up isolation and structure elucidation, which resulted in the identification of three new pyrrolosesquiterpenes, glaciapyrroles A (**1**), B (**2**), and C (**3**), as reported herein.



NPS008187 is an actinomycete that was isolated from a marine sediment collected in Alaska. Analysis of the near full length 16S rRNA sequence indicated that the strain is a *Streptomyces* sp. Two unique extracts were generated from fermentation broths of the producing strain, both of which contained compounds with the UV profiles of interest (λ_{max} 335, 285 (sh) nm). The first crude extract, obtained by extracting the culture broth with EtOAc, was subjected to flash C18 chromatography followed by reversed-phase HPLC to obtain glaciapyrrole A (**1**) with an isolated yield of only 0.2 mg/L and glaciapyrrole B (**2**) in even lesser yield (0.05 mg/L). The second crude extract was obtained by adding XAD-16 resin to the fermentation culture, filtering the culture broth to recover the cell mass and XAD-16 resin, and extracting the cell mass−resin with EtOAc. This second crude extract was then chromatographed by reversed-phase HPLC to obtain glaciapyrrole C (**3**) in an isolated yield of 0.05 mg/L.

Glaciapyrrole A (**1**) was obtained as a glassy semisolid ($[\alpha]^{22}_D$ +16.8 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (ϵ) 333 (14400), 285 (sh) (6700) nm; IR (NaCl) ν_{max} 3400, 3270, 2970, 1635, 1608, 1572, 1450, 1400, 1314, 1114, and 1061 cm^{-1}). A HRESI-TOF-MS measurement established the molecular formula as $C_{19}H_{27}NO_4$, indicating 7 degrees of unsaturation. The ^{13}C NMR and HMQC spectra confirmed the presence of 19 carbons, including one ketone carbonyl (δ_C 182.2 (C-6)), four carbons with chemical shifts and multiplicities that agreed well with a substituted pyrrole (δ_C 135.4 (C-2), 126.6 (C-5), 117.5 (C-3), 111.2 (C-4)), four olefinic carbons (δ_C 123.5 (C-7), 149.7 (C-8), 131.2 (C-9), 138.0 (C-10)), four aliphatic carbons bearing oxygen (δ_C 78.9 (C-11), 86.7 (C-12), 88.6 (C-15), 72.3 (C-16)), two aliphatic methylene carbons (δ_C 34.7 (C-13), 27.8 (C-14)), and four methyl groups (26.3 (C-17), 25.1 (C-20), 23.9 (C-19), 21.4 (C-18)). The pyrrole, further supported by the proton NMR data (Table 1), the ketone, and the two double bonds inferred from four olefinic carbon signals accounted for 6 degrees of unsaturation, requiring one additional ring. Since four of the carbons could be assigned to the pyrrole, we proposed that the remaining 15 carbons, including four methyl groups, comprised a sesquiterpenoid side chain; this proposal was ultimately confirmed through a series of COSY and HMBC correlations (Figure 1). Within the sesquiterpene side chain, several substructures were defined, including two olefinic spin systems (H-7, H-8, H_3-18 and H-9, H-10, H-11) that were joined by HMBC correlations from H-9 to C-8 and C-18, effectively creating a methyl-pentadienol. In addition, a tetrahydrofuran ring substituted at the 2- and 5-positions with a methyl group

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Table 1. ^{1}H NMR Assignments for Glaciapyrroles A (**1**), B (**2**), and C (**3**)

atom no.	**1** δ_H [a] int., mult, *J* (Hz)	**2** δ_H [a] int., mult, *J* (Hz)	**3** δ_H [b] int., mult, *J* (Hz)
1-NH			11.77 1H, br s
3	6.97 1H, br dd, 3.8, 1.3	6.97 1H, br d, 3.8	7.04 1H, br m
4	6.22 1H, dd, 3.8, 2.2	6.22 1H, dd, 3.8, 2.1	6.20 1H, dt, 3.8, 2.2
5	7.05 1H, br dd, 2.2, 1.3	7.05 1H, m	7.09 1H, m
7	6.67 1H, br s	6.67 1H, br s	6.75 1H, br s
9	7.83 1H, d, 16.1	7.81 1H, d, 16.1	7.97 1H, d, 16.1
10	6.23 1H, dd, 16.1, 6.6	6.31 1H, dd, 16.1, 6.5	6.03 1H, dd, 16.1, 7.6
11	4.12 1H, dd, 6.6	4.00 1H, d, 6.5	3.38 1H, d, 7.6
13	2.13 1H, m	1.56 1H, m	1.64 1H, m
	1.62 1H, ddd, 11.6, 8.2, 3.1	1.44 1H, m	1.47 1H, m
14	1.87 1H, m	2.08 2H, m	2.05 2H, m
	1.82 1H, m		
15	3.82 1H, dd, 9.5, 6.0	5.11 1H, br t, 7.2	5.10 1H, br t, 7.2
17	1.16 3H, s	1.65 3H, br s	1.65 3H, s
18	2.10 3H, br s	2.11 3H, s	2.06 3H, br s
19	1.15 3H, s	1.14 3H, s	1.26 3H, s
20	1.14 3H, s	1.59 3H, br s	1.57 3H, br s

[a] δ_H values referenced to internal solvent for CD_3OD at 3.31 ppm. [b] δ_H values referenced to internal solvent for DMSO-d_6 at 2.50 ppm.

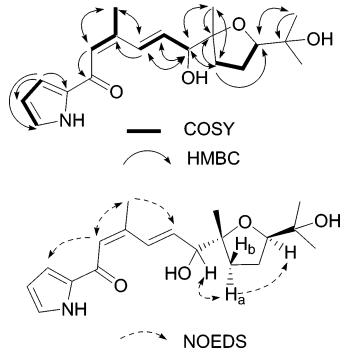

Figure 1. Key COSY, HMBC, and NOEDS correlations establishing the structure of **1**.

and a terminal dimethylcarbinol was delineated through a series of COSY and HMBC correlations (H-13 and H-19 to C-12; H-13 and H-17 to C-15; H-15 to C-20). The tetrahydrofuran (THF) was joined to the pentadienol via corroborative HMBC correlations from H-11 to C-12 and H-13 to C-11. The characterization of the sesquiterpenoid side chain was completed by joining the C-7/C-8 olefin to the ketone carbonyl through an HMBC correlation from H-7 to C-6. While H-7 was not correlated to C-2 in the HMBC spectrum, a correlation was observed at H-3 upon irradiation of H-7 in an NOEDS experiment, establishing the connectivity between the pyrrole and its sesquiterpene side chain.

For compound **1**, the geometries of the C-7/C-8 and C-9/C-10 double bonds were established through analysis of NOEDS experimental data and proton−proton coupling constants (Figure 1). Irradiation of the H_3-18 methyl proton resulted in enhancements at H-7 and H-10, supporting assignment of the *Z*-geometry for the C-7/C-8 olefin, while the H-9/H-10 coupling constant (J = 16.1 Hz) was consistent with the *E*-geometry. The relative stereochemistry of the THF ring was established by NOEDS analysis. Irradiation of one of the H_2-13 methylene protons at δ_H 2.13 (H_a-13) resulted in enhancements at H-15 and H-11, indicating that H-15 and the pentadienol side chain at C-12 are on the same side of the THF ring. Therefore, the C-19 methyl group and the terminal dimethylcarbinol groups must be on the other side of the THF ring. It was not possible to independently irradiate the H_3-17, H_3-19, and H_3-20 methyl protons due to near degeneracy; however, we note that simultaneous irradiation of all three methyl groups resulted in enhancement of H_b-13.

Table 2. ^{13}C NMR Assignments for Glaciapyrroles A (**1**), B (**2**), and C (**3**)[a]

atom no.	**1** δ_C [b] mult.	**2** δ_C [b] mult.	**3** δ_C [c] mult.
2	135.4 qC	135.4 qC	N.D.
3	117.5 CH	117.5 CH	115.9 CH
4	111.2 CH	111.2 CH	109.8 CH
5	126.6 CH	126.6 CH	125.7 CH
6	182.2 qC	182.2 qC	179.5 qC
7	123.5 CH	123.4 CH	122.6 CH
8	149.7 qC	149.9 qC	146.8 qC
9	131.2 CH	131.3 CH	132.4 CH
10	138.0 CH	138.0 CH	132.8 CH
11	78.9 CH	79.7 CH	62.3 CH
12	86.7 qC	75.5 qC	63.1 qC
13	34.7 CH_2	39.7 CH_2	37.9 CH_2
14	27.8 CH_2	23.0 CH_2	23.3 CH_2
15	88.6 CH	125.9 CH	123.7 CH
16	72.3 qC	132.1 qC	131.2 qC
17	26.3 CH_3	25.9 CH_3	25.4 CH_3
18	21.4 CH_3	21.4 CH_3	20.6 CH_3
19	23.9 CH_3	22.6 CH_3	16.4 CH_3
20	25.1 CH_3	17.7 CH_3	17.5 CH_3

[a] Multiplicities were determined by HSQC; N.D.: not detected. [b] δ_C values referenced to internal solvent for CD_3OD at 49.00 ppm. [c] δ_C values obtained through HSQC and HMBC and referenced to internal solvent for DMSO at 39.50 ppm.

Glaciapyrrole B (**2**) was also obtained as a glassy semisolid. Its molecular formula was established as $C_{19}H_{27}NO_3$ from a HRESI-TOF-MS measurement, requiring 7 degrees of unsaturation. While the spectral data for **2** were similar to those of **1** (Tables 1 and 2), the ^{13}C NMR spectrum contained two olefinic carbons in place of two carbons bearing oxygen. In addition, two methyl proton signals were downfield shifted (δ_H 1.59, s, H_3-20 and δ_H 1.65, s, H_3-17), while one of the methyl carbon signals was shifted upfield (δ_C 17.7, C-20). Together, these data suggested that the sesquiterpene side chain of **2** terminated in a typical dimethyl olefin. Complete analysis of the COSY, HMQC, and HMBC spectra indicated that **2** represented a C-11/C-12 diol. Although the isolation of glaciapyrrole B (**2**) from the EtOAc extraction of the whole fermentation broth is described, **2** was also detected in the resin fermentation condition and may be derived from epoxy derivative glaciapyrrole C (**3**) during the extraction and isolation process.

Glaciapyrrole C (**3**) was isolated from the resin extraction condition with limited recovery because of its instability. Its molecular formula was established as $C_{19}H_{25}NO_2$ based

on a HRESI-TOF-MS measurement, requiring 8 degrees of unsaturation. The spectral data for compound **3** compared favorably with that of **2**, with the exception that H-11 was shifted upfield from δ_H 4.00 to 3.38. To account for this observation, together with the additional degree of unsaturation and the loss of one oxygen atom when compared to **2**, compound **3** must be the epoxide.

Compounds **1**, **2**, and **3** were produced in low yield (0.05−0.2 mg/L); however, extracts of NPS008187 contained known diketopiperazines **4**, **5**, and **6**[3] in larger quantities (1−2 mg/L).

There are numerous natural products of mixed biosynthesis that comprise terpenoid substructures joined to aromatic ring systems of various types, such as phenols, quinones, coumarines, and flavonoids.[4−10] Pyrroles have been incorporated into a variety of natural products, yet their appearance among terpenes is rare.[4] While a number of pyrrolopolyenes have been reported,[11−13] to our knowledge, the only previously described pyrroloterpene natural product is pyrrolostatin (**7**),[14] which bears a carboxylic acid group at C-2 and a geranyl group at C-4. Thus, the three new pyrrolosesquiterpenes described herein are unique in nature. Compound **2** represents the product of epoxide ring opening of **3**, while **1** is related to **2** by oxidation at C-16 and addition of C-12(O) to C-15 to form the tetrahydrofuran ring. To determine if glaciapyrrole A (**1**) had antitumor activity, the cytotoxicity of **1** was determined against a pair of tumor cell lines at concentrations up to 1 mM. In a single set of experiments, **1** inhibited both colorectal adenocarcinoma HT-29 and melanoma B16-F10 tumor cell growth with an IC_{50} value of 180 μM.

Experimental Section

General Experimental Procedures. The optical rotation was obtained from an Autopol-III automatic polarimeter. NMR spectra were collected using a 500 MHz Bruker Avance spectrometer using an inverse probe equipped with *x*,*y*,*z*-gradients, except for the ^{13}C NMR spectrum, which was acquired with a broad-band observe probe. Data were acquired at 298 K in CD_3OD or DMSO-d_6. Mass spectra were acquired using a Micromass Q-Tof2 mass spectrometer with ES+ ionization. HRESI spectra were referenced using a poly(ethylene glycol) polymer mixture, which was co-injected during acquisition as an internal accurate mass standard. The reported UV spectral data were obtained from analytical HPLC analysis of the purified compound using an Agilent HP1100 HPLC equipped with an Agilent PDA detector (the mobile phase was a mixture of ACN and H_2O) as well as a Beckmann Coulter DU 640 spectrophotometer. The reduction of resazurin in the in vitro cytotoxicity assays was measured using a Fusion microplate fluorometer from Packard Bioscience.

Biological Material. Strain NPS008187 was isolated from a marine sediment sample collected from Alaska. Close to full length 16S rRNA sequence analysis of strain NPS008187 indicated that it is a *Streptomyces* sp. The culture was deposited on February 11, 2004, with the American Type Culture Collection (ATCC) in Rockville, MD, and assigned the ATCC patent deposition number PTA-5810.

In Vitro Cytotoxicity Assay. The cytotoxicity assays were performed essentially as described in the literature.[15] Briefly, the adherent cells were plated in 96-well plates and allowed to attach for 24 h at 37 °C. Serially diluted glaciapyrrole A (**1**) was added in triplicate to cells at concentrations ranging from 7.8 μM to 1 mM. Cells treated with a final concentration of 0.25% (v/v) DMSO served as the vehicle control. Cell viability was assessed 48 h later by measuring the reduction of resazurin with a fluorometer. The IC_{50} values were calculated in XLFit 3.0 software (ID Business Solutions Ltd) using a sigmoidal dose−response model.

Fermentation and Extraction of 1 and 2. Seed culture for the production of glaciapyrroles A (**1**) and B (**2**) was prepared from frozen stock culture grown in seed medium consisting of the following per liter of seawater: starch, 10 g; yeast extract, 4 g; and peptone, 2 g. The seed culture was incubated at 28 °C for 3 days on a rotary shaker operating at 250 rpm. The seed culture was inoculated into the production medium having the same composition as the seed medium. The production culture was incubated at 28 °C for 7 days on a rotary shaker operating at 250 rpm. The culture broth (10 L) was extracted with 10 L of EtOAc. The extract was dried in vacuo. The dried extract was then processed for the recovery of the compounds **1** and **2**.

Fermentation and Extraction of 3. Seed culture for the production of glaciapyrrole C (**3**) was prepared and grown as detailed above. The seed culture was inoculated into the production medium consisting of the following per liter of seawater: starch 5 g; Hydro Solubles, 4 mL; Menhaden fish meal, 2 g; kelp powder, 2 g; and chitosan, 2 g. The production culture was incubated at 28 °C for 4 days on a rotary shaker operating at 250 rpm. Sterile XAD-16 resin (~2 g) was added to each flask. The flasks were returned to the shaker and incubated at 28 °C and 250 rpm for an additional 3 days. The culture broth was filtered through cheesecloth to recover the cell mass and XAD-16 resin. The cell mass−resin was extracted with 10 L of EtOAc. The extract was dried in vacuo. The dried extract was then processed for the recovery of the compound **3**.

Purification of 1, 2, and 3. The crude extract (4.2 g) of NPS008187 containing glaciapyrroles A (**1**) and B (**2**) was dissolved in EtOAc, and the polar components were removed by partitioning with water. The EtOAc-soluble portion was concentrated (3.3 g) and chromatographed on a flash C18 column (15 cm × 40 mm ID) using a water/MeOH step gradient of 40%, 50%, 60%, 75%, and 100% MeOH. The known diketopiperazines **4**−**6** eluted in 50% and 60% MeOH fractions. Compounds **1** and **2** eluted in 75% MeOH. The glaciapyrrole-enriched fraction was further separated by HPLC using a C18 column (Phenomenex Luna, 10 μm, 25 cm × 21.2 mm i.d.) at a flow rate of 14.5 mL/min with a mobile phase gradient of 60% ACN/H_2O for 14 min, 60% to 70% ACN over 14 min to obtain semipure compounds **1** and **2**. These semipure compounds were further purified by HPLC using a C18 column (ACE 5 C18-HL, 25 cm × 10.6 mm i.d.) at a flow rate of 3 mL/min with a mobile phase gradient of 35% ACN/65% H_2O to 80% ACN over 14 min to obtain pure glaciapyrrole A (**1**; 0.2 mg/L) and B (**2**; 0.05 mg/L). The crude extract (196 mg) containing glaciapyrrole C (**3**) was dissolved in MeOH (3.92 mL), and 200 μL aliquots of this solution were sequentially injected onto the HPLC column (Phenomenex Luna, 10 μm, C18, 60 cm × 21.2 mm i.d.) at a flow rate of 14.5 mL/min with a solvent gradient of 10% to 80% ACN in 7 min, 80% to 100% in 1 min, 100% ACN for 5 min. The fraction enriched in **3** was further purified using semipreparative HPLC with a C18 column (ACE 5 C18-HL, 25 cm × 10.6 mm i.d.) at a flow rate of 3 mL/min with a solvent gradient of 50% to 80% MeOH/H_2O in 12 min, 5 min at 80% MeOH, 80% to 100% MeOH in 1 min, followed by 14 min at 100% MeOH to obtain glaciapyrrole C (**3**; 0.05 mg/L) as a pure compound.

Glaciapyrrole A (1): glassy semisolid; see text for $[\alpha]_D$, UV, and IR data; ^1H NMR (CD_3OD), see Table 1; ^{13}C NMR (CD_3OD), see Table 2; HRESIMS *m/z* 334.2016 [M + H] (calcd for $C_{19}H_{28}NO_4$, 334.2018).

Glaciapyrrole B (2): glassy semisolid; UV (ACN/H_2O) λ_{max} 335, 285 (sh) nm; ^1H NMR (CD_3OD), see Table 1; ^{13}C NMR (CD_3OD), see Table 2; HRESIMS *m/z* 318.2082 [M + H] (calcd for $C_{19}H_{28}NO_3$, 318.2069).

Glaciapyrrole C (3): glassy semisolid; UV (ACN/H_2O) λ_{max} 335, 285 (sh) nm; ^1H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 2; HRESIMS *m/z* 322.1792 [M + Na] (calcd for $C_{19}H_{25}NO_2Na$, 322.1783).

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