Thiasporines A–C, Thiazine and Thiazole Derivatives from a Marine-Derived Actinomycetospora chlora

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Supporting Information

ABSTRACT: Thiasporine A (1), the first natural product with a S-hydroxy-4H-1,3-thiazin-4-one moiety, along with two new thiazole derivatives, thiasporines B and C (2 and 3), were isolated from the marine-derived Actinomycetospora chlora SNC-032. The structures of 1–3 were established on the basis of comprehensive spectroscopic analysis and chemical methods. Thiasporine A showed cytotoxicity against the nonsmall-cell lung cancer cell line H2122 with an IC_{50} value of 5.4 μM.

Marine actinomycetes are prolific producers of biologically active natural products. This unique habitat has led to the abundant chemical diversity of metabolites, which provides a foundation for the discovery of promising drug lead compounds. Among all known marine microbial secondary metabolites, over half were produced by actinomycetes. From this resource, more than 400 new active secondary metabolites have been isolated. Some of them, represented by abyssomycin C, diazepinomicin, salinosporamide A, and the marinomycins, are potent antibiotics and possess novel structures. Many biologists and chemists have focused on exploiting biologically and structurally interesting compounds from marine microbes. As part of our ongoing research on structurally novel and bioactive compounds from marine-derived actinomycetes, 6500 microbial natural product fractions were screened against a panel of 17 comprehensively annotated non-small-cell lung cancer cell lines. The results showed that a series of natural product fractions demonstrated selective activity against a subset of these lines at 5 μg/mL. Analysis of an active fraction from the strain Actinomycetospora sp. SNC-032 by LC-UV-MS revealed it contained metabolites with similar UV absorptions at 230, 284, and 385 nm. This fraction showed selective cytotoxicity against the HCC44 cell line, while not showing activity against any of the other 16 cell lines. Bioassay-guided chemical investigation resulted in the isolation of a new thiazine and two new thiazole derivatives, which we have named thiasporines A–C (1–3). A known dithiopyrrolone, thiolutin, presenting antibacterial, antifungal, and anticaner properties, was also isolated. Thiasporine A (1) possessed a unique S-hydroxy-2-phenyl-4H-1,3-thiazin-4-one core, which is the first example of a naturally occurring compound possessing this skeleton. Thiasporine A exhibited moderate cytotoxicity against non-small-cell lung cancer cell line H2122 with an IC_{50} value of 5.4 μM, but no activity against HCC366, A549, and HCC44 cell lines.

Marine-derived bacterium SNC-032 was isolated from a sediment sample collected from a mangrove swamp in Vavau, Tonga (18°36′45″ S, 173°59′29″ W), and was isolated on a media prepared from rabbit manure. Analysis of its 16S rRNA sequence revealed SNC-032 to have greater than 99% identity to Actinomycetospora chlora TT071-57T. After identification of an active fraction in the library, a large-scale (10 L) shake fermentation was carried out to obtain sufficient material for full chemical and biological analysis of the metabolites. The excreted metabolites were collected using XAD-7-HP resin, and the resulting extract was separated by a combination of solvent/solvent extraction and reversed-phase flash chromatography to give fractions that showed cytotoxicity. Final purification by Sephadex LH-20 and gradient reversed-phase HPLC gave thiasporines A (1, 0.8 mg), B (2, 3.2 mg), and C (3, 1.8 mg).

Thiasporine A (1) was obtained as a white, amorphous powder. Its molecular formula was assigned as C_{15}H_{16}N_{2}O_{2}S from the [M + H]^{+} peak at m/z 221.0380 in the HRESIMS spectrum, which requires 8 degrees of unsaturation. The ^{13}C NMR resolved 10 carbon signals, which were classified by HMBC spectra as five olefinic or aromatic methine carbons and five quaternary carbons (Table 1). The proton signals at δ_H 7.51 (1H, d, J = 7.9), 7.10 (1H, t, J = 8.1), 6.79 (1H, d, J = 8.1), and 6.56 (1H, t, J = 7.8), along with their contiguous COSY signals.
Table 1. 1D and 2D NMR Data for Compound 1 in DMSO-

| no. | δ_C | δ_H mult. (J in Hz) | COSY | HMBC |
|-----|-----|---------------------|------|------|
| 1   | 114.4, C | 7.20, brs | 1, 3 |
| 2   | 146.5, C | 7.30, t (8.1) | 3, 5 |
| 3   | 116.1, CH | 6.79, d (8.1) | 4, 1, 5 |
| 4   | 130.2, CH | 7.10, t (8.1) | 3, 5 |
| 5   | 115.2, CH | 6.56, t (7.8) | 4, 6 |
| 6   | 128.4, CH | 7.51, d (7.9) | 5 |
| 7   | 164.4, C | | |
| 8   | 118.7, CH | 7.66, s | 7, 11 |
| 9   | 164.2, C | | |
| 10  | 157.7, C | 7.20, brs | 1, 3 |

aSpectra were recorded at 600 MHz for 1H and 100 MHz for 13C using the corresponding solvent residual signal as internal standard.

Table 2. 1H and 13C NMR Data for Compounds 2 and 3 in DMSO-d_6.

| no. | δ_C | δ_H mult. (J in Hz) | COSY | HMBC |
|-----|-----|---------------------|------|------|
| 1   | 113.8, C | 7.20, brs | 1, 3 |
| 2   | 147.0, C | 7.30, t (8.1) | 3, 5 |
| 3   | 111.0, CH | 6.79, d (8.3) | 4 |
| 4   | 131.9, CH | 7.33, td (8.5, 1.3) |
| 5   | 115.1, CH | 6.67, td (8.0, 1.0) |
| 6   | 129.2, CH | 7.66, dd (7.9, 1.4) |
| 7   | 168.8, C | 8.35, s |
| 8   | 125.8, CH | 8.35, s |
| 9   | 147.6, C | 8.35, s |
| 10  | 162.0, C | 8.35, s |
| 11  | 157.7, C | 7.20, brs |

aSpectra were recorded at 600 MHz for 1H and 100 MHz for 13C using the corresponding solvent residual signal as internal standard.

The COSY correlation of 2-NHCH3 (δ_H 2.92, 3H, d, J = 4.8) to 2-NH (δ_H 8.47, 1H, q, J = 4.8) and the key HMBC correlations from 2-NHCH3 (δ_H 2.92, 3H, d, J = 4.8) to C-2 and from 2-NH (δ_H 8.47, 1H, q, J = 4.8) to C-1 and C-3 (Figure 1). The remaining 1H and 13C signals (δ_H/C in Table 2) were consistent with the thiazole-4-carboxylic acid moiety (Figure S2). Moreover, the HMBC correlations from H-9 to C-7, C-10, and C-12 further confirmed this unit. The HMBC correlation from H-6 to C-7 indicated that the thiazine moiety connected aniline to thiasporine A (1) via a single bond between C-1 and C-7. There is a CAS registry entry for a synthetic compound containing the 5-hydroxy-2-phenyl-4H-1,3-thiazin-4-one core (# 1135682-81-3); however, there are no references associated with this entry.

Thiasporine B (2) was isolated as a white, amorphous powder and assigned the molecular formula C_{14}H_{14}N_{2}O_{3}S on the basis of the HRESIMS peak at m/z 235.0542 [M + H]⁺. The 1H NMR signals at δ_H 7.66 (1H, dd, J = 7.9, 14.4), 7.33 (1H, td, J = 8.5, 1.3), 6.79 (1H, d, J = 8.3), and 6.67 (1H, td, J = 8.0, 1.0) and their COSY correlations indicated that a 1,2-disubstituted phenyl nucleus was present in compound 2 (Table 2). The C = O and NHCH3 substitution was determined by the COSY correlation of 2-NHCH3 (δ_H 2.92, 3H, d, J = 4.8) to 2-NH (δ_H 8.47, 1H, q, J = 4.8) and the key HMBC correlations from 2-NHCH3 (δ_H 2.92, 3H, d, J = 4.8) to C-2 and from 2-NH (δ_H 8.47, 1H, q, J = 4.8) to C-1 and C-3 (Figure 1). The remaining 1H and 13C signals (δ_H/C in Table 2) were consistent with the thiazole-4-carboxylic acid moiety (Figure S2). Moreover, the HMBC correlations from H-9 to C-7, C-10, and C-12 further confirmed this unit. The HMBC correlation from H-6 to C-7 indicated that C-1 was connected with C-7.

The molecular formula of thiasporine C (3) was assigned to C_{14}H_{14}N_{2}O_{3}S based on the HRESIMS data, which was 14 mass units (one CH2) more than that of 2. Analysis of the 1H and 13C NMR spectra revealed that the methoxy signals at δ_C 52.2 appeared in compound 3. Thus, compound 3 was determined to be the methyl ester analogue of 2. Furthermore, the COSY and HMBC correlations confirmed this structure (Figure 1). A plausible biogenetic pathway for thiasporines A–C (1–3) (Scheme 2) begins by amide bond formation, which is followed by an intramolecular condensation of the antranilic acid moiety and cysteine to yield a hydroxy-thiazolidinecarboxylic acid ring system (intermediate a), which can undergo dehydration to produce the 4,5-dihydro-5-thiazoledicarboxylic acid system (ring b). Oxidation of intermediate b and

Scheme 1. Methylation of 1 with TMS–CHN2.

Figure 1. Key correlations for the structural assignment of 1–3.
Scheme 2. Plausible Biosynthetic Pathway of 1–3

![Scheme 2](image)

subsequent N-methylation of the aniline would give 2. More interesting is the biosynthetic conversion to 3 from a mangrove swamp in Vava'u, Tonga (18°36'45" S, 173°59'29" W). The sediment samples were collected under permits from the Tonga Ministry of Agriculture and Food, Forests and Fisheries, in conjunction with Dr. Peter Northcote (Victoria University, Wellington, NZ). The isolation media was prepared by boiling 100 g of rabbit manure in 1 L of seawater for 1 h and filtering, and the subsequent supernatant was added to 15 g of agar. Analysis of the 16S rRNA sequence of SNC-032 revealed 99% identity to *Actino-

**Experimental Section**

**General Experimental Procedures.** UV spectra were recorded on a Shimadzu UV-1601 UV–vis spectrophotometer. 1H and 2D NMR spectroscopic data were recorded at 600 MHz in CD3OD or DMSO-d6 solution on a Varian System spectrometer. 13C NMR spectra were acquired at 100 MHz on a Varian System spectrometer. 1H and 13C NMR, see Table S1; HRESIMS m/z 221.0380 [M + H]+ (calcd for C9H12N2O5S, 249.0692). In order to go from intermediate b to the final 5-hydroxy-4H-1,3-thiazin-4-one ring system in 1 requires a ring expansion and oxidation to give intermediate c. Finally, enolation would yield 1.

The active fraction showed selective cytotoxicity against the non-small-cell lung cancer cell line HCC44, so compounds 1–3 were evaluated for their cytotoxicity against the HCC44 cell line and another three non-small-cell lung cancer cell lines (HCC366, A549, and H2122). Compound 1 showed modest cytotoxicity against the cell line H2122 with an IC50 value of 5.4 μM, which was puriﬁed by reversed-phase HPLC (Phenomenex Luna, C18, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 20% to 100% CH3CN (0.1% formic acid) over 15 min to yield compound 1 (0.8 mg, tR = 13.7 min). Fractions 4 (83.9 mg) and 5 (75.0 mg) were combined and then recrystallized from MeOH to give thiolatnin (69.7 mg). The mother liquor of the recrystallization was separated by Sephadex LH-20, eluting with MeOH, to give 10 fractions. Subfraction 3-9 (8.6 mg) was puriﬁed by reversed-phase HPLC (Phenomenex Luna, C18, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 20% to 100% CH3CN (0.1% formic acid) over 20 min to yield compound 2 (3.2 mg, tR = 17.5 min). Fractions 6 (29.1 mg) and 7 (37.0 mg) were combined and then separated by Sephadex LH-20, eluting with MeOH, to give eight fractions. Subfraction 6-5 (9.4 mg) was puriﬁed by reversed-phase HPLC (Phenomenex Luna, C18 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 30% to 100% CH3CN (0.1% formic acid) over 25 min to afford compound 3 (1.8 mg, tR = 21.5 min).

**Thiasporine A (1):** white, amorphous powder; UV (MeOH) λmax (log e) 232 (4.08), 282 (3.62), 359 (3.58) nm; 1H and 13C NMR, see Table S1; HRESIMS m/z 221.0380 [M + H]+ (calcd for C9H12N2O5S, 249.0692).

**Thiasporine B (2):** white, amorphous powder; UV (MeOH) λmax (log e) 227 (4.04), 284 (3.70), 384 (3.68) nm; 1H and 13C NMR, see Table S1; HRESIMS m/z 235.0542 [M + H]+ (calcd for C11H13N2O5S, 253.0536).

**Thiasporine C (3):** white, amorphous powder; UV (MeOH) λmax (log e) 231 (4.11), 284 (3.71), 385 (3.66) nm; 1H and 13C NMR, see Table S1; HRESIMS m/z 249.0696 [M + H]+ (calcd for C12H13N2O5S, 267.0692).

**Methylation of 1 with TMS–CHN2.** To a solution of 1 (0.4 mg) in MeOH (anhydrous, 0.4 mL) was added 150 μL of TMS–CHN2 (2.0 M in Et2O) until a yellow color persisted upon addition. After allowing it to stir for 2 h, the solvent was removed via a stream of N2, and the reaction mixture was analyzed by LC/MS. The reaction product was purified by reversed-phase HPLC (Phenomenex Luna, C18 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system (solvents: A H2O + 0.1% formic acid; B CH3CN + 0.1% formic acid; gradient: 0 min: 30% B; 15 min: 100% B; 25 min: 100% B) to yield compound 1a (0.2 mg, tR = 14.9 min, 47% yield). Compounds 1a: white powder; 1H NMR (600 MHz, CD3OD) δ 8.28 (s, 1H, H-9), 7.61 (dd, J = 7.9, 1.4 Hz, 1H, H-6), 7.18 (dd, J = 8.4, 8.1, 1.1 Hz, H-6), 6.85 (dd, J = 8.4, 1.0 Hz, 1H, H-3), 6.67 (dd, J = 8.1, 8.1, 1.1 Hz, H-1, H-5), 3.94 (s, 3H, 10-OCH3) (ESIMS m/z 257.0 [M + Na]+).

**Cytotoxicity Assays.** Cell lines were cultivated in 10 cm dishes (Corning, Inc.) in NSCLC cell culture medium: RPMI1640-glutamine medium (Invitrogen, Inc.), 1000 U/mL penicillin (Invitrogen, Inc.), 1 mg/mL streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.). Cell lines were grown in a humidified environment in the presence of 5% CO2 at 37 °C. For cell viability assays, HCC366, A549, HCC44, and H2122 cells (60 μL) were plated individually at a density of 1200, 750, and 500 cells/well, respectively.
in 384-well microtiter assay plates (Bio-one; Greiner, Inc.). After incubating the assay plates overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 50 μM to 1 nM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer Glo reagent (Promega, Inc.) was added to each well (10 μL of a 1:2 dilution in NSCLC culture medium) and mixed. Plates were incubated for 10 min at room temperature, and luminescence was determined for each well using an Envision multimodal plate reader (PerkinElmer, Inc.). Relative luminescence was determined using the Assay Analyzer and Condoseo units were normalized to the untreated control wells (cells plus DMSO only). Data were analyzed using the Envision multimodal plate reader (PerkinElmer, Inc.). Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only). Data were analyzed using the Assay Analyzer and Condoseo modules of the Screener Software Suite (GeneData, Inc.) as described previously.13

**ASSOCIATED CONTENT**

1 Supporting Information
HRESIMS and NMR spectra for compounds 1–3 and 1a. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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**DEDICATION**

Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California—San Diego, for his pioneering work on bioactive natural products.

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