WS9326H, an Antiangiogenic Pyrazolone-Bearing Peptide from an Intertidal Mudflat Actinomycete

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Supporting Information

ABSTRACT: WS9326H (1), a new cyclic peptide, was isolated from a mudflat-derived Streptomyces strain. Based on analysis by 1D/2D NMR, UV spectroscopy, and mass spectrometry, compound 1 was determined to have the gross structure of a cyclic heptapeptide bearing an unprecedented pyrazolone ring connected to a D-arabinofuranosyl via an amide bond. The absolute configuration of 1 was established by multistep chemical derivatizations, comprehensive NMR, and LC/MS analyses of the derivatives and quantum mechanics-based computational methods. WS9326H (1) displayed significant antiangiogenesis activity.

Since the seminal discovery of the anticancer drug candidate salinomycin from the marine obligate actinomycete Salinispora tropica in the early 2000s,1 actinobacteria from marine habitats have been known for their ability to synthesize unique bioactive organic molecules with inspiring chemotypes.2 In our search for structurally and biologically novel compounds from actinomycetes inhabiting diverse marine environments, we discovered a new marine lasso-peptide, sungsanpin, from a sea sand-derived Streptomyces sp.3 A n dd i l a c t o n e-t e t h e r epseudodimeric peptides, alanine and 4-(2-nicrocyclopropyl)-propenylproline,7 ongoing detailed examination of the metabolites produced by this strain found a unique molecule with a maximum UV absorption at 280 nm and a molecular ion [M + H]+ at m/z 1313 by LC/MS analysis. Initial dereplication of the detected compound did not match any previously reported compounds, thus leading us to focus on the large-scale production of this molecule (1), so it could be characterized.

WS9326H (1) was purified as a white powder and provided an [M + Na]+ ion at m/z 1335.5570 in HR-FAB mass spectrometry. The observed exact mass was consistent with the molecular formula of C63H84N12O19 (calcd [M + Na]+ at m/z 1335.5878) and the 1H and 13C NMR data of 1 (Table S1). Its 1H NMR spectrum showed signals for 11 exchangeable protons (δH 12.79, 11.70, 10.55, 10.0, 9.81, 9.56, 9.54, 8.89, 8.72 (2H), and 8.53) in the downfield region (below 8 ppm), which could be amide NHs or phenolic OHs, thus suggesting the compound is peptide derived. The 13C NMR spectrum revealed that WS9326H (1) contains 11 amide/ester carbonyl signals (δC 176.9, 174.5, 174.3, 173.8, 173.6, 173.4, 172.7, 171.4, 170.8, 170.7, and 166.8) and 14 carbons in the region characteristic of α-carbonyl or oxygenated carbons (75.3, 74.9, 72.7, 72.5, 70.8, 65.2, 63.2, 62.8, 59.9, 58.7, 57.7, 56.3, 53.4, and 52.6), which are consistent with the peptide-derived structure indicated by the 1H NMR spectrum.

The 2D structure of WS9326H (1) was partially assigned by comparison of its 1D and 2D NMR spectra with those of WS9326A, a previously obtained abundant metabolite from the bacterial strain Streptomyces sp. SNM55. Comprehensive analysis of the 2D NMR spectra of 1, including COSY, TOCSY, HSQC, and HMBC experiments, revealed two threonine, a Me tyrosine (2,3-didehydro-Tyr), a leucine, a phenylalanine, an
asparagine, and a serine residue, which are also common to the aforementioned known peptide, WS9326A. The sequence of the amino acids was determined to be Thr-ΔMeTyr-Leu-Phe-Thr-Asn-Ser, which is identical to that of WS9326A. The HMBC correlation of H-26/C-61 closed the heptapeptide macrocyclic ring by connecting Thr to Ser through an ester linkage. This cyclic peptide ring accounted for 18 of the 28 double bond equivalents (two aromatic rings, one olefinic double bond, eight carbonyl carbons, and a macrocyclic ring) deduced from its molecular formula.

However, these seven residues account for only C_{40}H_{53}N_{8}O_{12} out of the molecular formula of I, C_{63}H_{84}N_{12}O_{19}. Thus, the structure of approximately one-third (C_{23}H_{31}N_{4}O_{7}) of the ring was constructed based on COSY correlations and 1H NMR data, allowing the construction of three additional double bond equivalents. Comprehensive analysis of the 1D and 2D NMR data allowed the construction of three additional partial structures (Figure 1). Consecutive COSY correlations from H-1 to H-10 revealed the C-10 spin system, accounting for a C_3 chain moiety. An ortho-substituted aromatic ring was constructed based on COSY correlations and 1H-1H coupling constants of the spin system spanning H-5 to H-8. This ring was confirmed by the HMBC correlations from H-6 and H-8 to C-4 and from H-5 and H-7 to C-9. The HMBC correlation of H-10/C-9 connected the C_3 chain from C-10 to C-14 to the C-9 position of the aromatic ring. The H_2/2H-3 COSY correlation and the H_2-2/C-1 HMBC correlation connected a C_3 chain from C-1 to C-3. H-3 displayed a three-bond HMBC correlation to C-5, thus constructing a 3-(2-[(pent-1-yl)-1-yl]phenyl)propanoic acid (PPPA) moiety, which requires one more substituent at C-3.

The COSY correlations from H-19 to H-23 constructed a C_5 chain between C-19 and C-23. Additionally, the correlations of H-20/20-OH, H-21/21-OH, H-22/22-OH, and H-23/23-OH assigned these hydroxyl protons to the C_5 chain. Further analysis of the COSY spectrum revealed that 18-NH was correlated with H-19, confirming the second partial structure to be a sugar alcohol moiety. This partial structure was supported by the TOCSY correlation from 18-NH to 23-OH (Figure 1).

Given that WS9326H bears seven amino acid units, a PPPA, and a sugar alcohol, the remaining molecular formula is only C_{40}H_{53}N_{8}O_{12} which must construct the last partial structure and contain four double bond equivalents. In the 1H NMR spectrum, there were three unassigned exchangeable protons at δ_{NN} 2.75 (2H) and 12.79. Further analysis of the 13C NMR spectrum revealed that two carbonyl peaks (δ_C 176.7 and 173.4), one double bond carbon (δ_C 159.6), and one fully substituted aliphatic carbon (δ_C 62.8) remained unassigned. Because two oxygen atoms were attributed to carbonyl functional groups along with their corresponding carbons (δ_C 176.7 and 173.4), the remaining exchangeable protons had to be bound to nitrogen atoms (NH at δ_NN 2.75 and NH at δ_NN 12.79).

Comprehensive analysis of the 2,3-bond HMBC correlations was used to assemble the structural fragments. The H-3 (δ_NN 4.72) aliphatic proton and 16-NH (δ_NN 2.75) showed HMBC correlation with C-16 (δ_C 62.8). The 16-NH2 protons also displayed clear HMBC cross peaks with the C-18 carbonyl carbon, which was connected to 18-NH based on the 18-NH/C-18 and H_{2-19}/18-C-18 HMBC correlations, indicating that the PPPA and the sugar alcohol were attached to the last partial structure. Furthermore, the HMBC correlations from H-3, 15-NH amide proton (δ_NN 12.79), and 16-NH₂ to the C-15 carbonyl carbon (δ_C 176.9) supported the connection between C-15 and C-16. However, even after complete analysis of the HMBC NMR data, there remained one unassigned quaternary carbon (C-17; δ_C 159.6) that showed no HMBC correlations to any protons. In addition, even after assigning the carbonyl groups, two double bond equivalents remained unaccounted for. Therefore, an imine functional group and an additional five-membered ring were assembled to generate a putative pyrazolone moiety (Figure 1). However, these connectivities were not directly supported by conventional NMR spectroscopic data.

To confirm the pyrazolone ring structure, an array of experiments including 15N-based NMR spectroscopic analysis and reducing the imine in the ring were performed. First, 15N HSQC analysis showed all the nitrogen signals, including the 16-NH₂ protons (δ_{NN} 2.72) bound to the pyrazolone ring and the downfield amide 15-NH (δ_NN 12.79) (Figure S8). Second, the C-17 imine was reduced selectively to the pyrazolidinone using NaBH₃CN as a hydride source.⁶ 17,17,17-Dihydro-WS9326H (2) was successfully yielded and analyzed by HSQC, COSY, and HMBC NMR spectroscopy (Figures S9–13). In the COSY spectrum of 2, the correlations between 15-NH/17-NH and 17-NH (δ_{NN} 6.55)/H-17 (δ_{NN} 5.48) were clearly observed, confirming the N–N bond of the pyrazolone ring in 1 (Figure 2). The HMBC correlations from 16-NH and 17-NH to C-17 (δ_C 52.7) in 2 also supported the assignment of C-17 as the carbon in the pyrazolone ring that was attached to C-16 (Figure 2). Finally, the planar structure of WS9326H (1) was determined to be a cyclic peptide with a unique acyl chain bearing a pyrazolone ring connected to a sugar alcohol motif via an amide bond, which is unprecedented.

Figure 1. Key 2D NMR correlations of the acyl chain bearing a PPPA, a pyrazolone, and a sugar alcohol.

Figure 2. Reduction of 1 and key COSY and HMBC correlations of 17,17-Dihydro-WS9326H (2).
Application of the advanced Marfe’s method successfully confirmed that the α-positions of the Leu, Asn, Ser, and two Thr units were in l-configurations and that the Phe residue in WS9326H (1) was in the d-configuration. The absolute configurations of the β-positions of the two Thr residues were analyzed by using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) derivatization (see the Supporting Information). LC/MS analysis of the GITC adducts of the two Thr units of I indicated that WS9326H (1) possesses both an l-Thr unit and an l-allo-Thr unit. These threonyne units were distinguished by J-based configuration analysis, thus placing the l-allo-Thr moiety between the D-Phe and l-Asn (Figure S28).

However, establishing the stereochemistry of the chiral carbons in the acyl chain of 1 was challenging. First, we designed four-step derivatizations of the sugar alcohol moiety (Scheme 1) that would allow us to complete the stereochemical determination. WS9326H (1) was derivatized with 2,2-dimethoxypropane, which yielded the desired 20,22-acetone of WS9326H (3). The 1H and 13C NMR chemical shifts of the two acetonide methyl groups of 3, based on its 1H and HSQC NMR spectra, were almost identical (δC, 25.6 and δH 1.31 for both), indicating an anti-relationship between the hydroxy groups at C-20 and C-22 (Figure S16). The relative configuration of the other secondary alcohol group at C-21 was determined based on the small 3JH21H22 value (4.5 Hz) and the large 3JH21H21 value (10.5 Hz) in conjunction with ROESY analysis, which assigned the relative configuration of the sugar alcohol moiety as 20S*, 21R*, 22S (Figure S30), thus assigning this sugar alcohol as D-arabinol.

To determine the absolute configurations of the C-3 and C-16 stereocenters, a quantum mechanics-based chemical shift analysis method, DP4 calculations, was utilized because chemical derivatization for stereochemical determination was not possible for these chiral centers. The four possible diastereomers (3R, 16R; 3R, 16S; 3S, 16R; and 3S, 16S) were proposed, and the 1H and 13C chemical shifts of a total of 120 conformers were calculated and averaged by their Boltzmann populations. Based on the comparison of the Boltzmann-averaged chemical shifts and the experimental chemical shifts, the DP4 analyses suggested the 3S, 16S configuration with 99.6% probability (Figure S31). The 3S, 16S configuration was also supported by the observed ROESY correlations in 1 (Figure S32).

As part of the biological evaluation of 1, its cytotoxic effects against human carcinoma cell lines A549, HCT116, SNU638, SK-HEP1, and MDA-MB231 were tested. However, WS9326H (1) did not exhibit significant cytotoxicity against the tested human cancer cells (IC50 > 10 μM). WS9326H (1) was also not active against the tested pathogens in the antibacterial and antifungal assays. In our subsequent bioactivity evaluations, antiangiogenesis was explored. Tumor angiogenesis has been known to play an important role in the growth of tumor cells and their metastasis because receiving nutrients and oxygen through new vessels can promote the growth of cancer cells. Therefore, the inhibition of tumor angiogenesis is regarded as a promising strategy for the treatment of cancer. The antiangiogenic activity of WS9326H (1) was evaluated by measuring the capillary tube formation in VEGF (vascular endothelial growth factor)-induced human umbilical vein endothelial cells (HUVECs). WS9326H (1) exhibited significant inhibition of endothelial cell network formation at 20 μM. This finding suggests that WS9326H (1) exhibits the antiangiogenic activity without cytotoxicity (Figures 3, S33, and S34; see the Supporting Information).

![Figure 3. Effect of WS9326H on capillary tube formation in endothelial cells under VEGF (20 ng/mL)-induced conditions.](image)

The WS9326 peptide family is a gradually growing class of nonribosomal peptides. The major metabolite, WS9326A, was originally reported in 1992 from Streptomyces violaceusniger as a tachykinin antagonist. More congeners (WS9326C-E) were discovered from another Streptomyces strain. WS9326D inhibited Brugia malayi asparaginyl-tRNA synthetase and killed the adult B. malayi parasite. Recently, WS9326F-G, truncated analogs of WS9326A with six amino acids (missing the seventh residue), were reported from Streptomyces asterosporus. These previously reported members of the WS9326 family of compounds bear identical 3-(2-((pent-1-en-1-yl)phenyl)-propanoic acid (PPPA) fragments, an acyl chain common to...
mannitol, as the only similar examples. Overall, our discovery of WS9326H's unprecedented structural motifs from mudfla-derived Streptomyces sp. demonstrates that marine actinomycetes could be a prolific reservoir of novel bioactive chemotypes.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b00546.

Detailed experimental procedures, 1H, 13C, and 2D NMR spectra (PDF)

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**Notes**

The authors declare no competing financial interest.

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