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Nyuzenamides A and B: Bicyclic Peptides with Antifungal and Cytotoxic Activity from a Marine-Derived *Streptomyces* sp.

Md. Rokon Ul Karim, Yasuko In, Tao Zhou, Enjuro Harunari, Naoya Oku, and Yasuhiro Igarashi*

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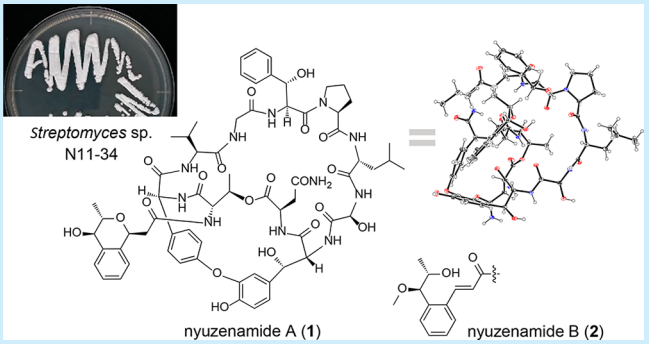
ABSTRACT: Two bicyclic peptides, nyuzenamides A (**1**) and B (**2**), were discovered from *Streptomyces* isolated from suspended matter in deep sea water collected in the Sea of Japan. Their structures were determined through nuclear magnetic resonance and mass spectrometry analyses in combination with X-ray crystallography and the chiral-phase gas chromatography–mass spectrometry method to comprise ten amino acid residues containing four unusual amino acids along with aromatic acyl units. Both compounds displayed antifungal activity against pathogenic fungi and cytotoxicity against P388 murine leukemia cells.



Microbial secondary metabolites have contributed to human welfare and the scientific community over many years. Among the bacterial genera, *Streptomyces* is undoubtedly the most productive genus, accounting for up to 9000 compounds. This figure is three times larger than that of secondary metabolites from *Aspergillus* or *Penicillum*, the most productive counterparts in eukaryotic microbes.[1] Although the average genome size of *Streptomyces* is 6 to 8 Mbp, not exceedingly larger than well-known secondary metabolite producers in prokaryotes such as myxobacteria (~13 Mbp) and cyanobacteria (~9 Mbp), its average number of biosynthetic gene clusters for secondary metabolites is quite large, typically more than 30.[2−5] Biosynthetic machineries for secondary metabolites in *Streptomyces* are much more diverse than other prokaryotic organisms, ranging from polyketides and peptides to isoprenoids, alkaloids, and additional structural classes derived from other pathways including carbohydrates and shikimates. Among these, modular type-I polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) have superior capability of generating complex and large molecules by virtue of their ability to utilize a range of small building blocks for chain elongation. In particular, recent advances in genetic engineering technology are expanding the possibility of generating new peptide scaffolds, rendering natural products of the peptide class more attractive targets for drug lead discovery than ever.[6]

In our continuing search for structurally novel secondary metabolites from marine actinomycetes to obtain new insights into drug design for antiinfective/anticancer agents, nyuzenamides A (**1**) and B (**2**), bicyclic peptides possessing several unusual structural features, were discovered from *Streptomyces* isolated from suspended matter in deep sea water of Toyama Bay, Japan (Figure 1). In this Letter, we describe the isolation, structure determination, and bioactivity of **1** and **2**.

The producing strain N11-34 was isolated from suspended matter collected from −311 m depth at the Nyuzen deep sea water pumping facility, Toyama, Japan.[7] The strain was identified as a member of the genus *Streptomyces* based on the 16S rRNA gene sequence analysis. The whole culture broth of strain N11-34 cultured in A16 liquid medium was extracted with 1-butanol, and the extract was fractionated by silica gel chromatography. Careful inspection of the fractions by high-performance liquid chromatography with a diode-array detector (HPLC-DAD) indicated the production of unknown metabolites showing UV spectra characteristic of non-conjugated benzene rings. UV-guided purification by reverse-phase HPLC yielded nyuzenamides A (**1**, 12.3 mg) and B (**2**, 14.5 mg) from a 3 L culture.

Nyuzenamide A (**1**) was isolated as a pale yellow, amorphous powder. The molecular formula was determined to be $C_{66}H_{81}N_{11}O_{20}$ on the basis of high-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESITOFMS) that gave a sodium adduct ion $[M + Na]^+$ at m/z 1370.5554 (Δ + 0.2 mmu). The UV spectrum in methanol, showing absorption bands at 203 and 273 nm, was indicative of the presence of benzene ring(s). The IR spectrum suggested the presence of NH or OH (3291 cm^{-1}) and

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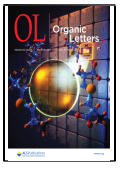

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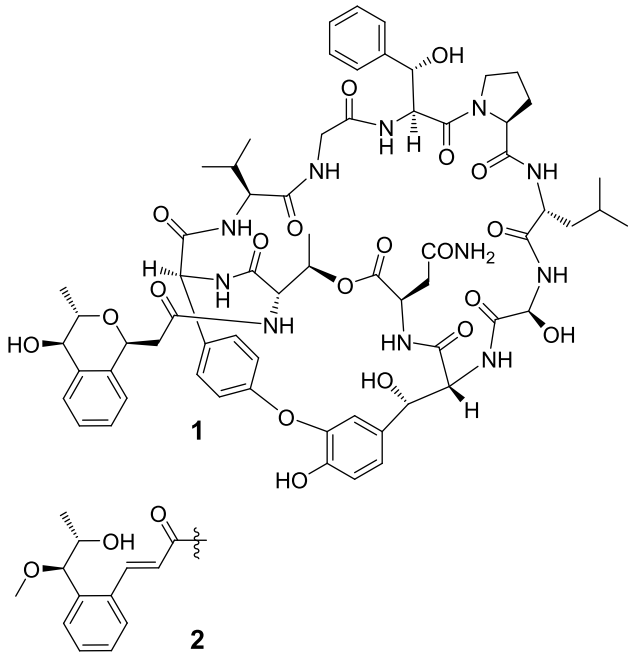

Figure 1. Structures of nyuzenamides A (**1**) and B (**2**).

carbonyl (1644 cm^{-1}) functionalities. Consistent with these analytical data, an initial inspection of 1D and 2D nuclear magnetic resonance (NMR) data revealed the presence of multiple aromatic protons/carbons (δ_H 6.40−7.39; δ_C 116.0−131.6), 12 carbonyl carbons (δ_C 168.4−175.9), and 9 amide protons (δ_H 7.51−9.10), respectively, connecting to α-methines/methylenes (δ_H 3.21−5.77; δ_C 42.7−71.4), suggesting the peptidic nature of this molecule (Table S1).

Analysis of correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectral data allowed the assignment of six common amino acids, Asx, Gly, Leu, Pro, Thr/*allo*-Thr, and Val (Figure 2), which accounted for five out of the nine signatures of α-amino acids. In parallel with the NMR analysis, chiral gas chromatography−mass spectrometry (GC-MS) analysis was conducted to confirm the amino acid composition and their absolute configurations. After heating in aqueous HCl, free amino acids in the hydrolysate of **1** were derivatized to trifluoroacetic acid (TFA) amide methyl esters by esterification in hydrochloric methanol followed by treatment with trifluoroacetic acid anhydride. The derivatized hydrolysates were analyzed by chiral-phase GC-MS, in comparison with authentic D- and L-amino acid derivatives, which established the presence of D-Asx, D-Leu, L-Pro, L-Thr, and L-Val (Figure S16). The remaining four pairs of amide protons and α-methines, on the contrary, were all assigned to be parts of oxygenated unusual α-amino acids, namely, hydroxyglycine (Hgy), β-hydroxyphenylalanine (Hpa), 4-hydroxyphenylglycine (Hpg), and α-hydroxytyrosine (Htr). Hydroxylation at the α- or β-positions of Hgy, Hpa, and Htr was evident from sequences of COSY correlations from amide protons to hydroxy protons (δ_H 5.73, 6.21, and 7.15), whereas the presence of an aromatic ring in Hpa, Hpg, and Htr was supported by HMBC correlations from ortho-aromatic protons to benzylic carbons (Figure 2). Oxygenation of the aromatic rings in Hpg and Htr was inferred by deshielded chemical shifts of carbons at the para and meta positions (δ_H 147.5, 147.8, and 160.3). Because both of the proton pairs symmetrically positioned on the phenoxy ring of Hpg (H4/H8 and H5/H7) were magnetically inequivalent, the ring rotation appeared to be severely restricted, which implied a connection of the phenolic oxygen to some other part of the molecule.

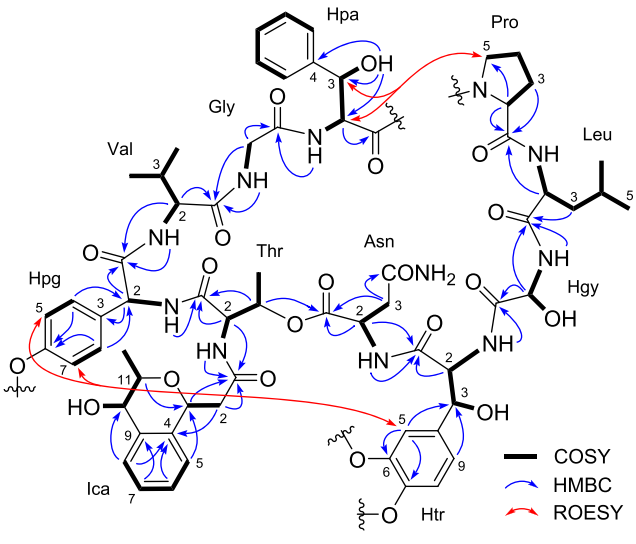

Figure 2. Key 2D NMR correlations for nyuzenamide A (**1**).

In addition to these amino acid residues, another component, a methyl- and hydroxy-substituted 2-(isochroman-1-yl)acetyl moiety (Ica), was assembled from the remaining three spin-coupled fragments and two nonprotonated aromatic carbons (δ_C 138.4, Ica-C4; 136.7, Ica-C9). A bundle of four aromatic protons (δ_H 7.12, 5.71, 6.92, and 7.29) had a doublet−triplet−triplet−doublet coupling pattern and correlated with both of these nonprotonated carbons in the HMBC spectrum, showing a typical signature of a 1,2-disubstituted benzene ring. The two substituents on this ring were an oxymethine (Ica-3) paired with a deshielded methylene (δ_H 2.64, 3.22/δ_C 41.5, Ica-2) and a hydroxy-substituted methine (Ica-10) linking to a methyl-substituted oxymethine (Ica-11), as supported by HMBC correlations from protons at both ends of the aromatic fragments to these methine carbons. Another HMBC correlation from Ica-H11 to Ica-C3 confirmed an ether bridge between Ica-C3 and Ica-C11, thus completing an isochroman core. Furthermore, the deshielded methylene and neighboring oxymethine protons showed HMBC correlations to a carbonyl carbon (δ_C 175.9, Ica-C1), which indicated a connection of the Ica unit to a hydroxy or amino functionality in the peptide chain.

The partial alignments of the amino acid residues were established by HMBC correlations from amide protons to the adjacent amide carbons, revealing Pro-Leu-Hgy-Htr-Asx and Thr-Hpg-Val-Gly-Hpa sequences. From the Thr β-proton was observed an HMBC correlation to a carboxyl carbon of Asx, which connected two peptide fragments via an ester linkage, whereas a correlation from the Thr α-proton to the Ica carbonyl carbon verified the acylation of the Thr amino group by the Ica unit.

The thus-assembled peptolide sequence contained one additional oxygen atom and one fewer H_3N molecule compared with the molecular formula, with six open termini of Pro-N, Htr-6-O, Htr-7-O, Asx-C4, Hpg-6-O, and Hpa-C1 remaining to be connected. Possible connectivities among these termini were partially given by a rotating frame

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Org. Lett. 2021, 23, 2109−2113

Overhauser effect spectroscopy (ROESY) spectrum (Figure S7). First, an amide linkage between Pro-N and Hpa-C1 was suggested by ROESY correlations Pro-H5/Hpa-H2 and Pro-H5/Hpa-H3 (Figure 2). In addition, ROESY correlations Hpg-H5/Htr-H5 and Hpg-H7/Htr-H5 implied an ether bridge between Hpg and Htr, which eliminated the excessive oxygen, as previously assumed. An exchangeable proton at δ_H 9.63, showing no COSY or HMBC correlation, was accordingly attributable to a phenolic proton at Htr-7-O, and the rest of the atoms, NH_2, were likely the components of a carboxamide blockade at the Asx-C4 terminus. No further information was available in the NMR data to validate these speculations. To our delight, however, during the storage of the NMR sample in a refrigerator, crystalline particles were formed, which were recovered and recrystallized from a mixture of MeOH–CH_2Cl_2 to provide colorless needles. The single-crystal X-ray analysis elucidated the gross structure of **1**, in which Pro and Hpa were connected through an amide bond, and Hpg and Htr were connected through an ether bridge at Hpg-C6 and Htr-C6, whereas a carboxamide group was present on Asx to form Asn (CCDC accession no. 2026824, Figure 3). The Flack

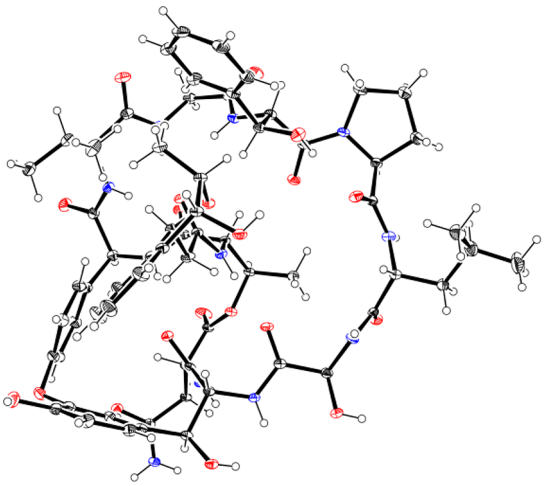

Figure 3. X-ray crystal structure of nyuzenamide A (**1**).

parameter (0.01) was small enough to verify the results from the chiral-phase GC-MS analysis and assign the remaining absolute configurations for the unusual amino acids and a nonpeptidic unit to be (*S*) for Hpg, (2*S*, 3*S*) for Hpa, (*S*) for Hgy, (2*R*, 3*S*) for Htr, and (3*S*, 10*R*, 11*S*) for Ica (Figure 1).

Nyuzenamide B (**2**) was also obtained as a pale yellow amorphous powder. Its molecular formula $C_{67}H_{83}N_{11}O_{20}$, determined by HR-ESITOFMS that displayed a sodium adduct ion $[M + Na]^+$ at *m*/*z* 1384.5706, was 14 amu larger than **1**, corresponding to the increment of a CH_2 fragment. Comprehensive analysis of 2D NMR spectra of **2** revealed that the ten amino acid residues, including four unusual amino acids, were totally identical to those in **1**, indicating a structural difference in the *N*-acyl substituent on Thr. The amino acid sequence was also proven to be the same by a series of HMBC correlations (Table S2).

Although HMBC correlations to support connectivities between Pro and Hpa and Hpg and Htr were lacking, the same set of ROESY correlations observed for **1** was also present, again supporting the amide linkage between Pro and Hpa and the ether linkage between Hpg and Htr (Figure S13). The remaining molecular parts for the nonpeptidic moiety, which eventually were assembled into a 2-(2-hydroxy-1-methoxypropyl)phenylpropenoyl group (Ppa), lacked the oxymethine and methylene units present in **1** and instead added methoxy (δ_H 2.69, s/δ_C 56.0) and disubstituted *E*-olefin (δ_H 6.84, d, *J* = 15.7 Hz; 8.03, d, *J* = 15.6 Hz) groups, suggesting the alteration of the substituents on the benzene ring (Figure 4). In fact, both

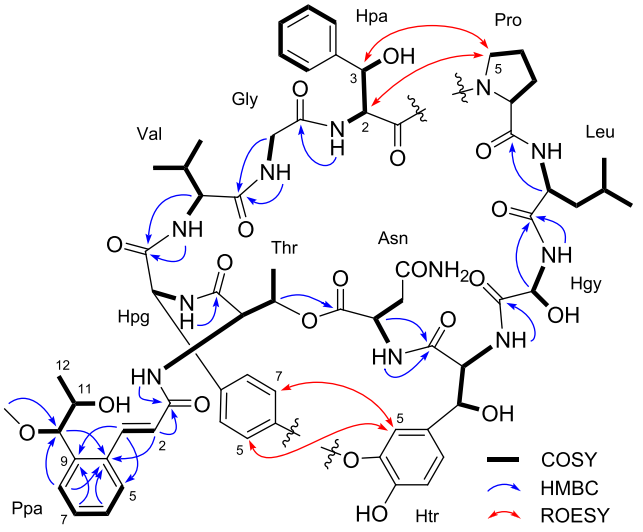

Figure 4. Key 2D NMR correlations for nyuzenamide B (**2**).

of the double-bond protons exhibited HMBC correlations to a carbonyl carbon at δ_C 168.8 (Ppa-C1) and a nonprotonated aromatic carbon at δ_C 135.4 (Ppa-C4), establishing a 2-propenoyl unit conjugated with a phenyl ring. The β-proton (Ppa-H3) of the acyl unit further correlated with another two aromatic carbons, one protonated (δ_C 127.1, Ppa-C5) and the other nonprotonated (δ_C 138.7, Ppa-C9), which, together with a four proton sequence traced in the COSY spectrum, supported a 1,2-disubstituted benzene structure. Finally, on the basis of the COSY and HMBC correlations, a 2-hydroxy-1-methoxypropyl group was placed at Ppa-C9 as the other substituent on the ring to complete the modified cinnamoyl unit as well as the gross structure of **2**. The absolute configurations of the common amino acids were identical to those in **1**, as determined by chiral-phase GC-MS analysis, and the same conclusion might be applicable to the rest of the amino acids in consideration of the global resemblance of the NMR spectra and biogenetic identity. The two chiral centers in the Ppa group were tentatively assigned to be 10R and 11S based on the interconvertible relationship of Ica and Ppa through β-elimination or Michael addition.

Nyuzenamides A (**1**) and B (**2**) were inactive against Gram-positive and -negative bacteria and a yeast but selectively inhibited the growth of filamentous fungi (Table S3). Compound **1** was more potent than **2** against plant and human pathogens, *Glomerella cingulata* NBRC5907 and *Trichophyton rubrum* NBRC5467, with a minimum inhibitory concentration (MIC) of 3.1 and 6.3 μg/mL, respectively. The MIC values of **2** against these fungi were 25 μg/mL. In addition, **1** and **2** exhibited cytotoxicity against P388 murine leukemia cells with IC_{50} of 4.9 and 6.2 μM (6.6 and 8.4 μg/mL, respectively).

In summary, the chemical investigation of a marine-derived *Streptomyces* strain N11-34 led to the discovery of two novel bicyclic peptides, nyuzenamides A (**1**) and B (**2**). The four

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Org. Lett. 2021, 23, 2109–2113

unusual amino acids in **1** and **2** are quite rare in nature. Hgy is known to be a component of spergualin, an antitumor antibiotic from *Bacillus*,[8] and dolyemycins, cyclic peptides from *Streptomyces*.[9] Hpa is a component of cyclic peptides from *Streptomyces*, dolyemycins, RP-1776,[10] ohmyungsamycins,[11] atratumycin,[12] and atrovimycin.[13] Htr is found in vancomycins from *Amycolatopsis*[14] and ralstonins from *Ralstonia solanacearum*.[15] Hpg is also a building block of vancomycins. The diphenyl ether bridge between aromatic amino acids is seen in some cyclic peptides such as vancomycins and seongsanamides,[16] but the linkage pattern between Htr and Hpg is unreported to date. The nonpeptidic units in **1** and **2**, oxygenated Ica and Ppa moieties, are also rare in nature. Similar structural parts, namely, the ortho-substituted cinnamates, are reported as an acyl decoration unit in RP-1776, atratumycin, atrovimycin, cinnapeptin,[17] and eudistamides,[18] all of which are the monocyclic depsipeptides from *Streptomyces*. The aromatic acyl portions in **1** and **2** are likely derived from the PKS pathway.[12,13,17,18] Starting from a propenylphenylpropenoate, dioxidation at the propenyl double bond (e.g., epoxidation/hydrolysis cascade) would give rise to the diol. Michael addition of the hydroxy group could lead to cyclic ether formation, thus yielding the Ica moiety. Further methylation of one hydroxy group could afford the Ppa moiety (Figure 5). Although many bicyclic peptides such as

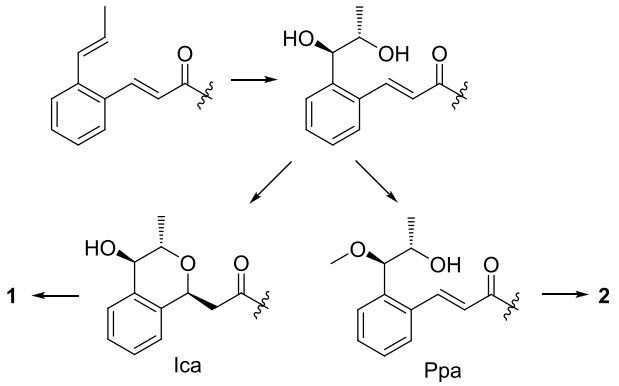

Figure 5. Plausible biosynthetic relationship between Ica and Ppa moieties.

seongsamides and salinamides[19] are known from nature, overall, **1** and **2** have no structural resemblance to any of the known natural peptides. The discovery of **1** and **2** expands the structural diversity of microbial peptides and provides new scaffolds for the development of new antifungal antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c00210.

Experimental details, UV, IR, and NMR spectra, and NMR data tables for **1**and **2** (PDF)

Accession Codes

CCDC 2026824 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

■ AUTHOR INFORMATION

Corresponding Author

Yasuhiro Igarashi − *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan;* orcid.org/0000-0001-5114-1389; Phone: +81-766-56-7500; Email: yas@pu-toyama.ac.jp; Fax: +81-766-56-2498

Authors

Md. Rokon Ul Karim − *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan;* orcid.org/0000-0002-1891-8894

Yasuko In − *Department of Physical Chemistry, Osaka University of Pharmaceutical Sciences, Takatsuki, Osaka 569-1041, Japan*

Tao Zhou − *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan;* orcid.org/0000-0002-6359-2598

Enjuro Harunari − *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan;* orcid.org/0000-0001-8726-0865

Naoya Oku − *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.orglett.1c00210

Notes

The authors declare no competing financial interest.

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