

Padanamides A and B, Highly Modified Linear Tetrapeptides Produced in Culture by a *Streptomyces* sp. Isolated from a Marine Sediment

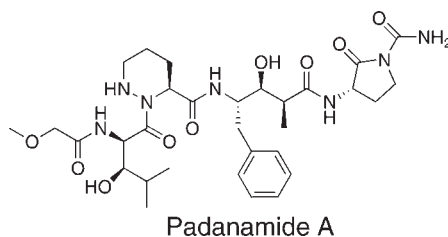
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ABSTRACT



Two highly modified linear tetrapeptides, padanamides A (1) and B (2), are produced by laboratory cultures of a *Streptomyces* sp. obtained from a marine sediment. Padanamide B is cytotoxic to Jurkat cells, and a chemical genomics analysis using *Saccharomyces cerevisiae* deletion mutants suggested that padanamide A inhibits cysteine and methionine biosynthesis or that these amino acids are involved in the yeast's response to the peptide.

Microorganisms living in the world's oceans represent a vast and largely unexplored resource of biodiversity that has the potential to yield new families of bioactive secondary metabolites that could be deployed in the challenging clinical battle against antibiotic-resistant microbial pathogens or other human diseases.¹ As part of an ongoing

program aimed at discovering new natural products produced by microorganisms isolated from marine habitats,² it was found that laboratory cultures of a *Streptomyces* sp.

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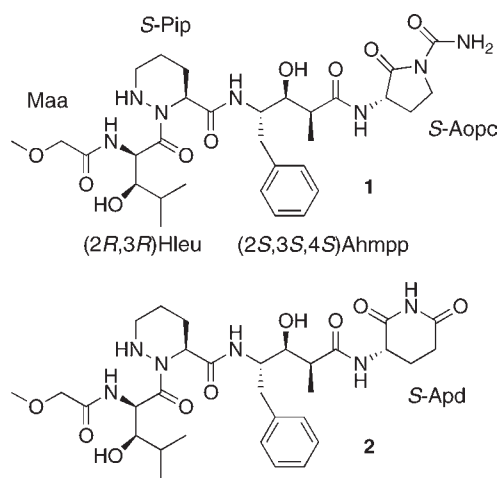
(1) Hughes, C. C.; Fenical, W. *Chem.—Eur. J.* **2010**, *42*, 12512–12525.

(2) (a) Carr, G.; Tay, W.; Bottriell, H.; Andersen, S. K.; Mauk, A. G.; Andersen, R. J. *Org. Lett.* **2009**, *11*, 2996–2999. (b) Desjardine, K.; Pereira, A.; Wright, H.; Matanaiho, T.; Kelly, M.; Andersen, R. J. *J. Nat. Prod.* **2007**, *70*, 1850–1853. (c) Barsby, T.; Kelly, M. T.; Andersen, R. J. *J. Nat. Prod.* **2002**, *65*, 1447–1451. (d) Barsby, T.; Kelly, M. T.; Gagne, S. M.; Andersen, R. J. *Org. Lett.* **2001**, *3*, 437–440. (e) Gerard, J.; Haden, P.; Kelly, M. T.; Andersen, R. J. *Tetrahedron Lett.* **1996**, *37*, 7201–7204.

(3) Isolate RJA2928 was identified as a *Streptomyces* sp. by analysis of its 16S rRNA sequence (GenBank accession number JF513115). It is 99% identical to *Streptomyces* sp. 1043 (EU864307) and *Streptomyces* sp. MJM4686 (EU603353); both strains were isolated from soil. It also showed 99% identity to *Streptomyces malaysiensis* strain Da08002 (EU595361).

(isolate RJA2928)³ obtained from marine sediment collected near the passage Padana Nahua in Papua New Guinea generated crude organic extracts that showed potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Bioassay-guided fractionation of the crude extract identified the known natural product 1-*O*-methyl-30-acetyl nigericin as the major source of antimicrobial activity.⁴

NMR data obtained for several chromatography fractions devoid of antibacterial activity showed signals indicative of highly modified peptides. Further NMR-guided HPLC purification of these fractions yielded the two new tetrapeptides, padanamides A (**1**) and B (**2**). Details of the isolation, structure elucidation, and biological activities of the padanamides are presented below.



Production cultures of RJA2928 were grown as lawns on solid agar marine medium (Supporting Information) at rt for 14 d. The combined cells and media from the solid agar culture were cut into small squares and extracted repeatedly with EtOAc. Concentration of the EtOAc extracts in vacuo gave a gummy brown residue that was partitioned between EtOAc and H₂O. Fractionation of the EtOAc soluble material using sequential application of Sephadex LH20 chromatography, open column step-gradient Si gel chromatography, and reversed-phase HPLC gave pure samples of padanamides A (**1**) (72 mg) and B (**2**) (11 mg).

Padanamide A (**1**) was obtained as an optically active viscous oil that gave a $[\text{M} + \text{Na}]^+$ ion in the HRESIMS at m/z 684.3328 appropriate for a molecular formula of C₃₁H₄₇N₇O₉, requiring 12 sites of unsaturation. ¹H/¹³C/gCOSY/gHSQC/gNHSQC/gNlrHMQC/gHMBC NMR data obtained for **1** (Table 1, Supporting Information) identified resonances that could be assigned to 31 carbon and 7 nitrogen atoms, in agreement with the HRESIMS measurement. Three exchangeable proton doublets at δ 7.43 (Hleu-NH), 7.58 (Ahmpp-NH), and 8.17 (Aopc-NH) showed one-bond correlations to nitrogen resonances at δ -264.3 (Hleu-N), -262.5 (Ahmpp-N), and -262.2 (Aopc-N), respectively, in the g¹⁵NHSQC spectrum and correlations to carbon resonances at δ 174.5 (Aopc-C-2),

174.1 (Ahmpp-C-1), 172.3 (Hleu-C-1), 170.4 (Pip-C-1), and 168.4 (Maa-C-1) in the gHMBC spectrum, with chemical shifts appropriate for amide carbonyls, consistent with a peptide structure for **1**.

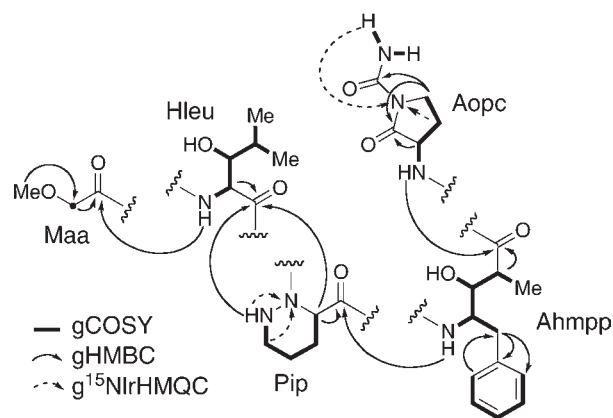


Figure 1. Selected 2D NMR correlations observed for padanamide A (**1**).

Detailed analysis of the NMR data recorded for padanamide A (**1**) identified the presence of 2-methoxyacetic acid (Maa), 3-hydroxyleucine (Hleu), piperazic acid (Pip), 4-amino-3-hydroxy-2-methyl-5-phenylpentanoic acid (Ahmpp), and 3-amino-2-oxopyrrolidine-1-carboxamide (Aopc) residues (Figure 1 and Supporting Information). These fragments accounted for all of the atoms and the 12 sites of unsaturation required by the molecular formula of **1**, indicating that padanamide A had to be a linear peptide. The sequence of **1** was established by the gHMBC correlations shown in Figure 1 and was confirmed by the tROESY NMR data (Supporting Information).

The absolute configuration of padanamide A (**1**) was determined by chemical degradation. Acid hydrolysis with 6 M HCl followed by derivatization with 1-fluoro-2,4-dinitrobenzene gave the degradation products **3**, **4**, and **5** that were obtained as pure compounds from the reaction mixture after separation by reversed-phase HPLC (Scheme 1).

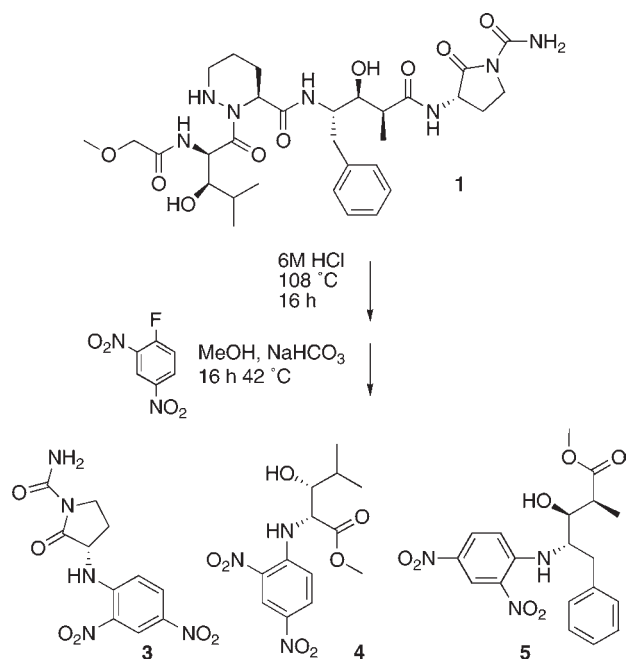
Each of the derivatives **3**, **4**, and **5** gave yellow crystals that were suitable for single-crystal X-ray diffraction analysis. The structures of compounds **3**, **4**, and **5** were determined using Cu K α radiation, so as to employ anomalous dispersion effects to determine each molecule's absolute configuration. Routine refinement of the Flack *X* parameter [**4**: 0.02(2) and **5**: -0.05(3)] showed that **4** had the 2*R*,3*R* configuration and **5** had the 2*S*,3*S*,4*S* configuration.⁵ Crystals of compound **3** were of marginal quality, so its absolute configuration could not be unambiguously determined on the basis of the refined Flack *X*

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(5) (a) Flack, H. D. *Acta Crystallogr., Sect. A* **1983**, *39*, 876–881. and (b) Bernardinelli, G.; Flack, H. D. *Acta Crystallogr., Sect. A* **1985**, *41*, 500–511.

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Scheme 1



parameter [−0.15(34)] alone. However, a statistical analysis of the Bijvoet pairs⁶ yielded a 97.5% probability that the absolute configuration at C-3A and C-3B is *S* and a 2.5% probability that the material is a racemic mixture. ORTEP diagrams generated for **3**, **4**, and **5** (Figure 2 and Supporting Information) confirmed the constitutions of the Aopc, Hleu, and Ahmpp residues that were assigned from the NMR analysis.

The authentic piperazic acid derivative **8** was obtained by treating a commercial racemic mixture of piperazic acid with Marfey's reagent, separation of the diastereomers **6** and **7** via reversed-phase HPLC, and methylation of **7** with TMSD (Scheme 2). Single-crystal X-ray diffraction analysis of **8** showed that the piperazic acid moiety in the molecule had the *S* configuration (see the Supporting Information). HPLC comparison of the authentic *R* and *S* piperazic acid Marfey's derivatives **6** and **7** with the material produced from standard Marfey's analysis of padanamide A (**1**) showed that the piperazic acid residue in the natural product had the *S* configuration.

Padanamide B (**2**) was obtained as an optically active viscous oil that gave a [M + Na]⁺ ion in the HRESIMS at *m/z* 669.3183 appropriate for a molecular formula of C₃₁H₄₆N₆O₉, which differs from the formula of **1** by the loss of NH but still requires 12 sites of unsaturation. The ¹H and ¹³C NMR spectra obtained for **2** showed a strong resemblance to the corresponding spectra obtained for **1**, differing primarily in the resonances assigned to the C-terminus residue. Thus, resonances assigned to the carboxamide functionality [δ 152.7 (C-6), −294.3 (N-7), 7.42/7.73 (H-7/H-7')], N-1 (δ −231.8), and the C-5 methylene [δ 3.44/3.71 (H-5/H-5'), 41.5 (C-5)] portions of the

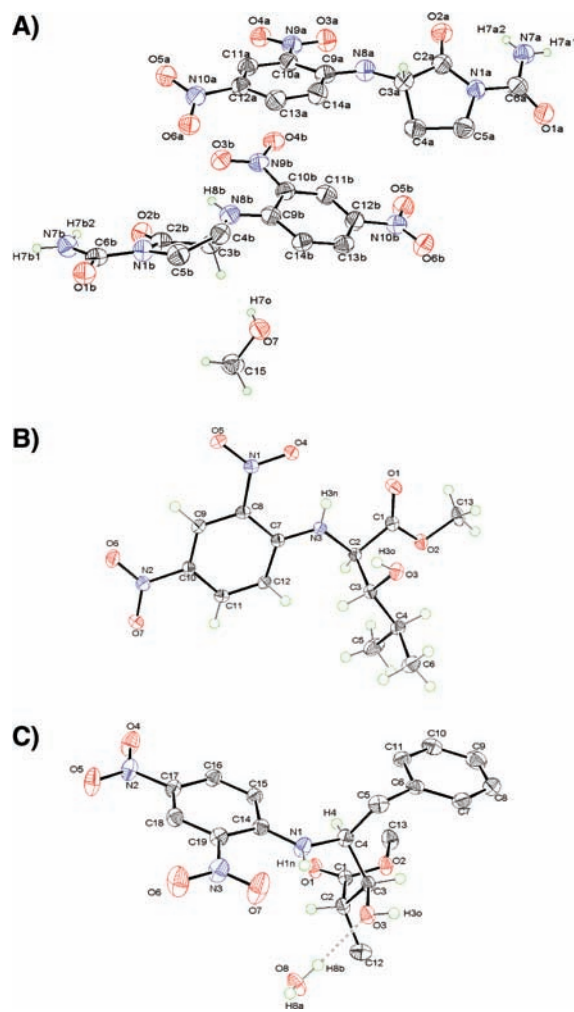


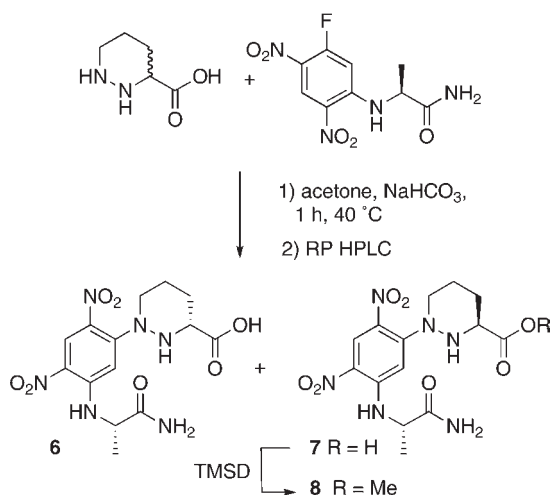
Figure 2. ORTEP diagrams for **3** (panel A), **4** (panel B), and **5** (panel C).

3-amino-2-oxopyrrolidine-1-carboxamide moiety in **1** were no longer present in the spectra of **2**. In their place, the ¹H/¹³C NMR spectra recorded for **2** contained resonances assigned to a more upfield aliphatic methylene [δ 2.48/2.72 (H-5/H-5'), 30.9 (C-5)], an amide carbonyl (δ 173.0, C-6), and an NH (δ 10.85, correlated to δ −208.2 in the g¹⁵NHSQC experiment).

The NH resonance at δ 10.85 showed HMBC correlations to carbonyl resonances at δ 172.7 (Apd-C-2) and 173.0 (Apd-C-6) to the methylene carbon resonance at δ 30.9 (Apd-C-5) and to a methine carbon resonance at δ 49.0 (Apd-C-3). This set of HMBC correlations was consistent with presence of an imide functionality incorporated into a 3-aminopiperidine-2,6-dione (Apd) residue. HMBC correlations observed between the C-1 amide carbonyl (δ 174.0) of the Ahmpp residue in **2** and both the Apd H-3 resonance (δ 4.45) and the Apd amide resonance (δ 8.09), showed that the Apd residue was present at the C-terminus of padanamide B (**2**).

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Scheme 2



Marfey's analysis⁷ of the 6 M HCl hydrolysate of **2** established that the absolute configuration at C-3 of Apd is *S*, the same as C-3 of the Aopc residue in **1**, by comparison with *R/S*-glutamic acid standards. The HPLC retention times for the Marfey's derivatives of Hleu, Pip, and Ahmpp in the trace of **2** were the same as **1**, confirming that the absolute configurations of **1** and **2** were identical at all the stereogenic centers.

Padanamide B (**2**) was cytotoxic to Jurkat T lymphocyte cells (ATCC TIB-152) *in vitro* with an IC₅₀ of 20 μg/mL, while padanamide A (**1**) was roughly 3-fold less active (IC₅₀ ≈ 60 μg/mL) in the same assay.

Chemical genomics has proven to be a useful technique in discovering the mode of action of drugs. It can also be used to detect interesting interactions between biological targets and natural products that do not manifest themselves as hits in bioassays designed to discover therapeutic agents. This technique involves analyzing genes that become sensitive or essential in the presence of applied compounds.^{8,9} To determine the chemical genomics profile of padanamide A, it was first checked for bioactivity against *Saccharomyces cerevisiae*. A very slight growth inhibition was detected using a drug-hypersensitive *S. cerevisiae* strain in rich media. Chemical genomic profiling requires only a minor growth inhibition for the assay, so it

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(9) Costanzo, M.; Baryshnikova, A.; Bellay, J.; Kim, Y.; Spear, E. D.; Sevier, C. S.; Ding, H.; Koh, J. L.; Y. Toufighi, K.; Mostafavi, S.; St. Prinz, J.; Onge, R. P.; VanderSluis, B.; Makhnevych, T.; Vizeacoumar, F. J.; Alizadeh, S.; Bahr, S.; Brost, R. L.; Chen, Y.; Cokol, M.; Deshpande, R.; Li, Z.; Lin, Z.-Y.; Liang, W.; Marback, M.; Paw, J.; San Luis, B.-J.; Shuteriqi, E.; Tong, A. H. Y.; van Dyk, N.; Wallace, I. M.; Whitney, J. A.; Weirauch, M. T.; Zhong, G.; Zhu, H.; Houry, W. A.; Brudno, M.; Ragibizadeh, S.; Papp, B.; Pal, C.; Roth, F. P.; Giaever, G.; Nislow, C.; Troyanskaya, O. G.; Bussey, H.; Bader, G. D.; Gingras, A.-C.; Morris, Q. D.; Kim, P. M.; Kaiser, C. A.; Myers, C. L.; Andrews, B. J.; Boone, C. *Science* **2010**, *327*, 425–431.

was possible to screen padanamide A with this drug hypersensitive strain. A modified version of the barcode sequencing assay¹⁰ was used to generate a chemical genomic profile of deletion mutants with altered abundance in the presence of padanamide A. Correlation analysis was then used to compare the chemical genomic profile with the genetic interaction database.⁹

The chemical genomic profile of padanamide A had significant overlap with the *CYS4* deletion mutant's genetic interaction profile ($p = 0.03$), and the correlation was driven largely by the genes *AAH1* and *MET32* (Supporting Information, Figure S11). *CYS4* is cystathionine β-synthase, which catalyzes the first committed step in cysteine biosynthesis, and *MET32* is a transcription factor that regulates methionine biosynthetic genes. Given these results, padanamide A was tested for its effect on sulfur amino acid biosynthesis with a series of minimal media assays (Supporting Information, Figure S12). Cells growing on media lacking cysteine and methionine have a reduced growth in the presence of padanamide A compared with the solvent control ($p < 0.001$). Addition of cysteine or methionine partially recovers the reduced growth, with the recovery from methionine alone significantly greater than cysteine alone ($p < 0.05$). Together these data suggest that padanamide A is involved in inhibition of sulfur amino acid biosynthesis or that these amino acids are involved in response to the peptide. Interestingly, padanamide B did not exhibit the same link to sulfur amino acid biosynthesis at the concentration where padanamide A was active.

Padanamides A (**1**) and B (**2**) are highly modified linear tetrapeptides that are notable for the complete absence of protein amino acids in their structures. The (2*S*,3*S*,4*S*)-Ahmpp residue found in the padanamides has been described in the literature as a synthetic building block used in the preparation of a library of bleomycin analogues, but it has not been previously encountered in a natural product.¹¹ Similarly, the (*S*)-3-amino-2-oxopyrrolidine-1-carboxamide (Aopc) and 3-aminopiperidine-2,6-dione (Apd) residues found at the C-termini of padanamides A (**1**) and B (**2**), respectively, are apparently unprecedented in naturally occurring peptides.

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Supporting Information Available. Experimental details, tables of NMR assignments for **1** and **2**, 1D and 2D NMR spectra for **1** and **2**, and details of X-ray diffraction analyses for **3**, **4**, **5**, and **8**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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