Organic LETTERS
Letter
pubs.acs.org/OrgLett

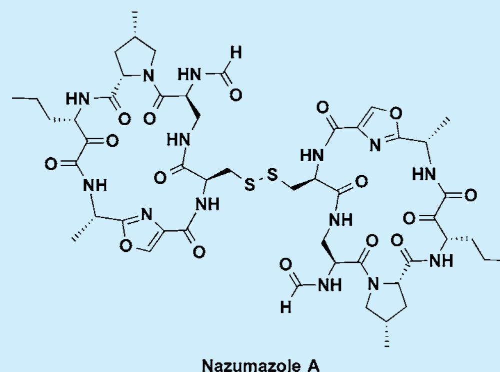
Nazumazoles A−C, Cyclic Pentapeptides Dimerized through a Disulfide Bond from the Marine Sponge *Theonella swinhoei*

Kazuya Fukuhara, Kentaro Takada,* Shigeru Okada, and Shigeki Matsunaga*

Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Supporting Information

ABSTRACT: A mixture of nazumazoles A−C (**1**−**3**) was purified from the extract of the marine sponge *Theonella swinhoei*. The mixture was eluted as an extraordinarily broad peak in the reversed-phase HPLC. The structures of nazumazoles were determined by interpretation of the NMR data and chemical degradations. Nazumazoles contain one residue each of alanine-derived oxazole and α-keto-β-amino acid residue. Nazumazoles exhibited cytotoxicity against P388 cells.



Marine sponges of the genus *Theonella* have been shown to be a rich source of structurally unique and biologically active secondary metabolites.[1,2] From *T. swinhoei* with a yellow interior (*T. swinhoei* Y) collected at Hachijo Island we have isolated more than 40 bioactive polyketides and modified peptides such as onnamides,[3] polytheonamides,[4] aurantosides,[5] and orbiculamides.[6] Recently, a symbiotic filamentous bacterium, *Candidatus* Entotheonella sp., was identified as the sole producer of a variety of natural products from this sponge.[7] We have analyzed the extracts of marine invertebrates by LCMS and found that the extract of *T. swinhoei* Y contained unidentified metabolites, among which was an extraordinarily broad peak in ODS-HPLC (Figure 1, Figure S1, Supporting Information). We successfully purified the peak, which exhibited cytotoxicity against P388 murine leukemia cells. We describe the isolation, structure elucidation, and biological activity of the constituents of the peak, nazumazoles A−C (**1**−**3**).

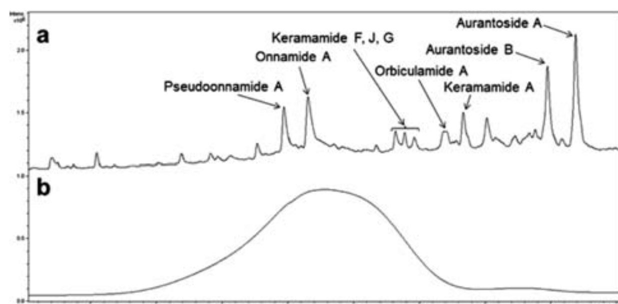


Figure 1. LCMS charts of the crude fractionof *T. swinhoei* Y: (a) UV chromatogram (integration of 210−400 nm) with assignments of some known compounds[3,5,6,8] and (b) MS chromatogram of a sum of the responses of the ions at *m*/*z* 1185, 1199, and 1213.

The MeOH extract of the sponge was partitioned between H_2O and Et_2O, and the organic layer was further partitioned between 90% MeOH and *n*-hexane. The H_2O and 90% MeOH fractions were combined and fractionated by ODS flash chromatography, gel permeation chromatography, gel permeation HPLC, and ODS-HPLC to afford nazumazoles A−C (**1**−**3**) as an inseparable mixture.

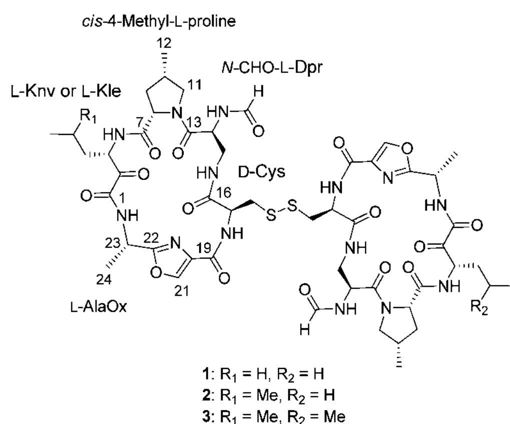
The HRESIMS of the mixture of nazumazoles A−C showed the molecular formulas of nazumazoles A, B, and C to be $C_{50}H_{68}N_{14}O_{16}S_2$, $C_{51}H_{70}N_{14}O_{16}S_2$, and $C_{52}H_{72}N_{14}O_{16}S_2$, respectively. Interestingly, **1**−**3** gave different molecular ion peaks in the ESIMS when the solvent of the sample and carrier liquid was altered from MeCN to MeOH or EtOH. For example, the protonated ion peaks of nazumazole C (**3**) were observed at *m*/*z* 1213, 1277, and 1305, respectively, when using MeCN, MeOH, and EtOH, indicating that two molecules of MeOH or EtOH were incorporated into the molecular ion, reminiscent of the FABMS data of cyclotheonamide A, in which alcohol adducts were formed at the α-keto-β-amino acid residue.[9]

^1H and ^{13}C NMR data (Table 1) suggested the peptidic nature of the molecules with several amide ^1H signals and carbonyl ^{13}C signals. Interpretation of the COSY, HSQC, and TOCSY spectra revealed the presence of Cys, 2,3-diaminopropionic acid (Dpr) and 4-methylproline (MePro) residues. The α-amino group of the Dpr residue was formylated as indicated by the HMBC correlations between H-14 (δ 4.76) and the formyl ^{13}C signals (δ 160.7) and between the formyl ^1H (δ 7.99) and C-14 (δ 50.5)

Received: April 9, 2015
Published: May 11, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acs.orglett.5b01020
Org. Lett. 2015, 17, 2646−2648



signals (Figure 2). There were ^{1}H NMR spin systems attributable to leucine and norvaline (Nva) residues, whose nitrogen substituted methine ^{1}H signals were correlated to a ^{13}C signal at δ 197.3 (197.2), suggesting the presence of α-ketoleucine (Kle) and α-ketonorvaline (Knv) residues.[10] The presence of a disubstituted oxazole ring was deduced from the HMBC data, in which a singlet aromatic ^{1}H signal (δ 8.54) directly coupled to a ^{13}C signal at δ 142.4 by 213 Hz was correlated to ^{13}C signals at δ 134.5 and δ163.5.[9] Further HMBC correlations from the H-23 (δ 5.01) and H-24 (δ 1.54) signals to the C-22 (δ 163.4) signal suggested that the oxazole ring was a part of an alanine-derived oxazole (AlaOx). At this moment, two amide carbonyl ^{13}C (δ 159.6 and 163.5) signals were left unassigned, one of which was in the α-keto-β-amino acid residue and the other attached to the AlaOx residue as inferred from a process of elimination.

The amino acid sequences of **1**−**3** were determined by HMBC and NOESY data (Figure 2). HMBC correlations between 3-NH/C-7, H-11α/C-13, H-14/C-13, H-15β/C-16, H-18β/C-16, and H-17/C-19 suggested the sequence of -Knv (Kle)-MePro-isoDpr-Cys-. The amide ^{1}H signal of the Cys residue was correlated to a ^{13}C signal at δ 159.6, and the nitrogen-substituted methine ^{1}H signal of the AlaOx residue was correlated to a ^{13}C signal at δ 163.5 in the HMBC spectrum. Therefore, the Cys residue was shown to be connected to the AlaOx residue via a carbonyl carbon and the AlaOx residue was linked to the Knv/Kle residue.

Because the sizes of the resulting pentapeptide fragments were about half of the molecular weights of nazumazoles A−C, we considered that these peptides were either cyclic decapeptides or cyclic pentapeptides dimerized through a disulfide bond. In order to clarify this issue, the mixture of **1**−**3** was reduced consecutively with $NaBH_4$ and DTT and subjected to alkylation with 4-vinylpyridine to afford a mixture of **4a** and **4b** and that of **5a** and **5b**, whose HRESIMS suggested that they were reduced cyclic pentapeptides with an *S*-pyridylethyl group.[11] In **4a** and **4b**, the α-carbon of Knv residue was reduced to a secondary alcohol (rKnv residue), whereas in **5a** and **5b** the pertinent residue was replaced by the reduced Kle (rKle) residue. Even though the structures of **4a**, **4b**, **5a**, and **5b** were confirmed by the 2D NMR data, each pair of diastereomers was inseparable by HPLC. Therefore, **1**−**3** were cyclic pentapeptides dimerized through a disulfide bond.

We then examined the mode of dimerization by analyzing the $NaBH_4$ reduction products of the mixture of nazumazoles A−C, which gave three sharp HPLC peaks (Figure S3, Supporting Information, **6**−**8**). The ESIMS and 2D NMR data demonstrated that the smallest congener (peak A, Figure S3) contained

Table 1. ^{1}H and ^{13}C NMR Data for the Mixture of Nazumazoles A (1)−C (3) in DMSO-d_6

residue	position	δ_C[a], type	δ_H[a] (*J*, Hz)
Knv	1	163.5,[b] C	
	2	197.2, C	
	3	54.6, CH	4.82, m
	4	31.4, CH_2	1.94, m
			1.56, m
	5	19.1, CH_2	1.48, m
			1.40, m
	6	13.3, CH_3	0.90, m
	3-NH		8.59, d (6.0)
Kle	1	163.5, C	
	2	197.3, C	
	3	53.3, CH	4.87, m
	4	38.0, CH_2	1.76, m
			1.46, m
	5	24.8, CH	1.78, m
	6	21.0, CH_3	0.90, m
	5-Me	22.9, CH_3	0.93, m
	3-NH		8.56, m
MePro	7	172.4, C	
	8	59.5, CH	4.44, m
	9	37.1, CH_2	2.42, m
			1.29, m
	10	33.2, CH	2.21, m
	11	53.9, CH_2	3.90, m
			2.94, m
	12	15.8, CH_3	0.99, d(6.4)
Dpr	13	167.0, C	
	14	50.5, CH	4.76, m
	15	38.7, CH_3	3.68, m
			3.45, m
	14-NH		7.98, m
	15-NH		7.48, m
	CHO	160.7, CH	7.99, s
Cys	16	170.6, C	
	17	52.7, CH	4.58, m
	18	39.1, CH_2	3.14, m
			2.71, m
	17-NH		7.81, m
AlaOx	19	159.6, C	
	20	134.5, C	
	21	142.4, CH	8.54, s
	22	163.5,[b] C	
	23	43.7, CH	5.01, m
	24	15.7, CH_3	1.54, d(6.9)
	23-NH		8.95, m

[a]Recorded at 150 MHz for ^{13}C and 600 MHz for ^{1}H at 40 °C. [b]Chemical shifts of C-1 and C-22 overlapped each other.

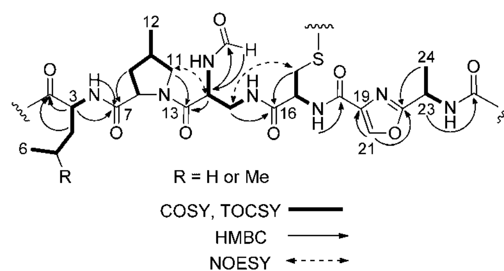
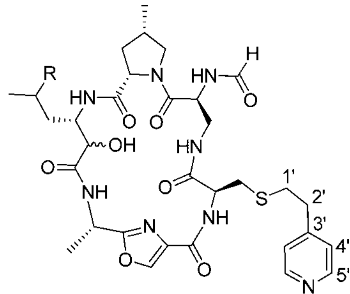


Figure 2. Key COSY, TOCSY, HMBC, and NOESY correlations in **1**−**3**.

DOI: 10.1021/acs.orglett.5b01020
Org. Lett. 2015, 17, 2646−2648

two rKnv residues, the second congener (peak B, Figure S3) contained one each of rKnv and rKle residue, and the largest congener (peak C, Figure S3) contained two rKle residues.[12]



4a and **4b**: R = H
5a and **5b**: R = Me

The absolute configurations of the amino acid residues in **1**−**3** were determined by Marfey's method.[13] Ala, Cys, Dpr, and MePro were liberated by standard acid hydrolysis. Knv and Kle residues were analyzed as Nva and Leu residues, respectively, by oxidation with H_2O_2. Two diastereomers of 4-methylproline were synthesized from *trans*-4-hydroxy-L-proline as described in the literature and used as standards.[14] Marfey's analysis with detection by ESIMS revealed the presence of L-Ala, D-Cys, L-Dpr, *cis*-4-methyl-L-proline, L-Leu, and L-Nva (Figures S30−S32, Supporting Information).

The mixture of nazumazoles A (**1**)−C (**3**) exhibited cytotoxicity against P388 murine leukemia cells with an IC_{50} value of 0.83 μM. The cytotoxicity was diminished when the ketone in **1**−**3** was reduced, whereas the cytotoxicity was lost by further reduction and alkylation of the thiol group (Table S8, Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

Description of experimental procedure and spectroscopic data of **1**−**8**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01020.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: atakada@mail.ecc.u-tokyo.ac.jp.
*E-mail: assmats@mail.ecc.u-tokyo.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products"[15] and JSPS KAKENHI Grant Nos. 25252037, 25712024, and 25660163 from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

■ REFERENCES

(1) Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1988**, *37*, 2162−2178.
(2) Winder, P. L.; Pomponi, S. A.; Wright, A. E. *Mar. Drugs.* **2011**, *9*, 2643−2682.
(3) (a) Sakemi, S.; Ichiba, T.; Kohmoto, S.; Saucy, T.; Higa, T. *J. Am. Chem. Soc.* **1988**, *1* (10), 4851−4853. (b) Matsunaga, S.; Fusetani, N.; Nakao, Y. *Tetrahedron* **1992**, *48*, 8369−8376.
(4) Hamada, T.; Matsunaga, S.; Yano, G.; Fusetani, N. *J. Am. Chem. Soc.* **2005**, *127*, 110−118.
(5) Matsunaga, S.; Fusetani, N.; Kato, Y.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 9690−9692.
(6) Fusetani, N.; Sugawara, T.; Matsunaga, S.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 7811−7812.
(7) Wilson, M. C.; Mori, T.; Ruckert, C.; Uria, A. R.; Helf, M. J.; Takada, K.; Gernert, C.; Steffens, U. A. E.; Heycke, N.; Schmitt, S.; Rinke, C.; Helfrich, E. J. N.; Brachmann, A. O.; Gurgui, C.; Wakimoto, T.; Kracht, M.; Crusemann, M.; Hentschel, U.; Abe, I.; Matsunaga, S.; Kalinowski, J.; Takeyama, H.; Piel, J. *Nature* **2014**, *506*, 58−62.
(8) (a) Kobayashi, J.; Sato, M.; Ishibashi, M.; Shigemori, H.; Nakamura, T.; Ohizumi, Y. *J. Chem. Soc., Perkin. Trans 1* **1991**, *10*, 2609−2611. (b) Itagaki, F.; Shigemori, H.; Ishibashi, M.; Nakamura, T.; Sasaki, T.; Kobayashi, J. *J. Org. Chem.* **1992**, *57*, 5540−5542. (c) Kobayashi, J.; Itagaki, F.; Shigemori, I.; Takao, T.; Shimonishi, Y. *Tetrahedron* **1995**, *51*, 2525−2532.
(9) Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053−7054.
(10) The presence of Kle and Knv residues was confirmed by the Marfey's analysis of the hydrolysate, which gave peaks for of DAA-derivatized rKnv (*m/z* 400) and DAA-derivatized rKle (*m/z* 414) (cf. the experimental section in the Supporting Information). This was confirmed by the HMBC data of **4a**, **4b**, **5a**, and **5b**.
(11) The reduction product of the mixture of **1**−**3** with DTT gave a broad HPLC peak in ODS-HPLC [*m/z* 594 and 608 $(M + H)^+$], and the two constituents were not separable.
(12) Each peak in Figure S2 (Supporting Information) contains four diastereomers which were not separable by ODS-HPLC. Each peak gave two sets of NMR signals depending on the configuration of the newly generated secondary alcohol.
(13) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(14) Murphy, A. C.; Mitova, M. I.; Blunt, J. W.; Munro, M. H. G. *J. Nat. Prod.* **2008**, *71*, 806−809.
(15) Ueda, M. *Chem. Lett.* **2012**, *41*, 658−666.

DOI: 10.1021/acs.orglett.5b01020
Org. Lett. 2015, 17, 2646−2648