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Thalassospiramides A and B, Immunosuppressive Peptides from the Marine Bacterium *Thalassospira* sp.

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ABSTRACT

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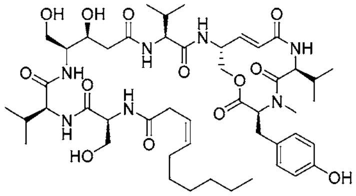

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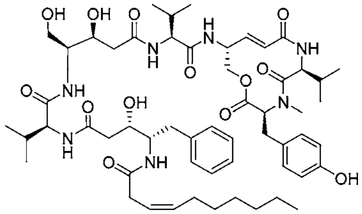

Two new cyclic peptides, thalassospiramides A and B (1 and 2), were isolated from a new member of the marine α-proteobacterium *Thalassospira*. The thalassospiramides, the structures of which were assigned by combined spectral and chemical methods, bear unusual γ-amino acids and show immunosuppressive activity in an interleukin-5 production inhibition assay ($IC_{50} = 5\ \mu M$ for thalassospiramide B).

Marine bacteria are now being exploited as a new source for novel secondary metabolites.[1] The presence of new actinomycete marine bacteria had not been appreciated until the application of precise phylogenetic analysis.[2] Chemical investigations of these new actinomycetes are now resulting in the discovery of novel secondary metabolites with pharmaceutical potential.[3] As part of a program to discover new marine bacterial taxa, we identified a new marine eubacterium, which is closely phylogenetically related to the α-proteobacterium *Thalassospira lucentensis*.[4] In culture, this α-proteobacterium (strain CNJ-328) produced two interesting peptides, thalassospiramides A and B (**1** and **2**). Here, we report the isolation, structural determination, and biological activity of these new compounds, the first secondary metabolites to be reported from *Thalassospira* spp.

Thalassospiramide A (**1**)[5] was isolated as a gum, which analyzed for the molecular formula $C_{48}H_{75}N_7O_{13}$ by high-resolution MALDI-TOF mass spectrometry coupled with ^1H and ^{13}C NMR spectral data (Table 1). The ^1H and gCOSY NMR spectra showed the typical features for peptides with six amide proton signals [δ_H: 9.24, 9.03, 8.86, 8.56, 8.48, 7.73], one *N*-methyl group [δ_H: 3.10], and seven α-amino protons [δ_H: 5.33, 5.19, 4.89, 4.73, 4.62, 4.53, 4.22]. The ^1H homonuclear *J*-resolved NMR spectrum revealed six doublet [δ_H: 0.9∼1.1] and one triplet methyl groups [δ_H: 0.79]. In addition to the general peptide features, the proton NMR spectrum showed four olefinic protons at δ 6.98, 6.91, 5.89, and 5.55, which are uncommon in ordinary peptides. Carbon-

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[1] (a) Jensen, P. R.; Fenical, W. *J. Indust. Microbiol. Biotechnol.* **1996**, *17*, 346. (b) Jensen, P. R.; Mincer, T. J.; Williams, P. G.; Fenical, W. *Antonie van Leeuwenhoek* **2005**, *87*, 43.

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[3] (a) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2003**, *42*, 355. (b) Buchanan, G. O.; Williams, P. G.; Feling, R. H.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Org. Lett.* **2005**, *7*, 2731. (c) Oh, D.-C.; Williams, P. G.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Org. Lett.* **2006**, *8*, 1021.

[4] (a) López-López, A.; Pujalte, M. J.; Benlloch, S.; Mata-Roig, M.; Rosselló-Mora, R.; Garay, E.; Rodríguez-Valera, F. *Int. J. System Evol. Microbiol.* **2002**, *52*, 1277. (b) The most closely related bacterium that has been identified is *Thalassospira lucentensis* (98% identity, AF358664), and thus CNJ-328 may represent a new *Thalassospira* species.

[5] Thalassospiramide A (**1**): oil; $[\alpha]_D$ −29 (*c* 0.07, CH_3CN); IR (neat) ν_{max} 3354, 2966, 1740, 1643, 1519 cm^{-1}; UV (CH_3CN) λ_{max} (log ϵ) 221 (4.3), 278 (3.3) nm; NMR spectral data, see Table 1 and Table S1 in the Supporting Information; HR-MALDI-TOFMS $[M + H]^+$ *m/z* 958.5528 ($C_{48}H_{76}N_7O_{13}$, calcd $[M + H]^+$ 958.5496).

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Table 1. NMR Spectral Data for **1** in Pyridine-d_5

C/H	$\delta_H{}^a$	mult (J in Hz)	$\delta_C{}^b$		HMBC
1			170.2	C	
2	4.22	dd (11.0, 4.5)	67.6	CH	1, 10
3a	3.58	dd (14.0, 4.5)	34.7	CH_2	2, 5, 9
3b	3.77	dd (14.0, 11.0)			2, 5, 9
4			129.3	C	
5	7.31	d (8.0)	131.0	CH	3
6	7.08	d (8.0)	116.6	CH	3
7			157.6	C	
7-OH	11.44	br s			
8	7.08	d (8.0)	116.4	CH	3
9	7.31	d (8.0)	130.7	CH	3
10	3.10	s	40.0	CH_3	1, 11
11			171.9	C	
12	4.53	dd (6.5, 4.0)	59.0	CH	11, 13
12-NH	7.73	d (6.5)			12, 13, 16
13	2.07	m	29.8	CH	
14	0.94	d (7.0)	20.2	CH_3	12, 13, 15
15	1.14	d (7.0)	17.1	CH_3	12, 13, 14
16			170.9	C	
17	6.91	m	126.8	CH	16, 19
18	6.98	m	139.6	CH	16, 19
19	5.19	br m	49.7	CH	17, 18
19-NH	8.56	d (7.5)			17, 18, 19, 21
20a	4.79	dd (11.0, 2.5)	64.0	CH_2	1, 18
20b	4.30	dd (11.0, 2.5)			18
21			171.8	C	
22	4.73	dd (8.0, 8.0)	60.0	CH	21, 22
22-NH	9.03	d (8.0)			22, 26
23	2.42	m	30.3	CH	21, 22, 25
24	0.94	d (7.0)	20.2	CH_3	22, 23, 24
25	1.02	d (7.0)	19.0	CH_3	22, 23, 24
26			172.7	C	
27a	3.10	dd (14.0, 2.5)	42.2	CH_2	26, 28, 29
27b	2.99	dd (14.0, 5.5)			26, 28, 29
28	5.09	br m	68.0	CH	
28-OH	6.95	m			
29	4.62	m	56.2	CH	
29-NH	8.48	d (9.0)			31
30	4.13	m	62.2	CH_2	
30-OH	6.61	br t (5.0)			
31			172.5	C	
32	4.89	dd (8.0, 7.0)	60.4	CH	31
32-NH	9.24	d (8.0)			32, 36
33	2.42	m	31.0	CH	31, 32, 34, 35
34	1.02	d (7.0)	20.1	CH_3	32, 33, 35
35	1.02	d (7.0)	18.7	CH_3	32, 33, 34
36			172.6	C	
37	5.33	td (7.5, 7.0)	56.4	CH	36
37-NH	8.86	d (7.5)			37, 39
38	4.26	m	63.0	CH_2	
38-OH	7.20	m			
39			171.9	C	
40	3.32	d (7.0)	35.5	CH_2	39, 41, 42
41	5.89	dtt (11.0, 7.0, 1.5)	123.1	CH	40, 43
42	5.55	dtt (11.0, 7.5, 2.0)	133.2	CH	40, 43, 44
43	1.98	m	28.0	CH_2	44
44	1.22	m	29.9	CH_2	45
45	1.12	m	29.4	CH_2	
46	1.10	m	32.2	CH_2	
47	1.13	m	23.1	CH_2	
48	0.79	t (5.0)	14.6	CH_3	46, 47

[a] 300 MHz. [b] 100 MHz.

13 NMR and gHSQC spectra displayed eight amide or ester carbonyl carbons, ten olefinic carbons, eight methines, and three methylenes bearing oxygen or nitrogen between 49 and 68 ppm, three aliphatic methines, eight aliphatic methylenes, one downfield methyl at 40.0 ppm, and seven upfield methyl signals. Analysis of gHSQC spectral data allowed all one-bond carbon and proton correlations to be assigned (Table 1).

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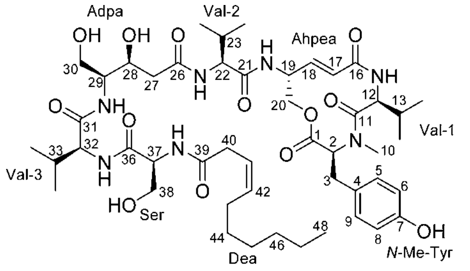

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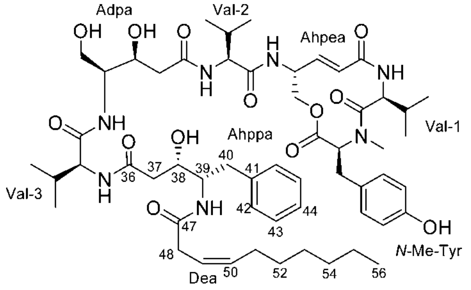

Interpretation of gCOSY, TOCSY, and gHMBC NMR data allowed eight distinct fragments to be constructed. These included unusual amino acids such as 4-amino-5-hydroxy-penta-2-enoic acid (Ahpea), 4-amino-3,5-dihydroxy-pentanoic acid (Adpa), as well as *N*-methyltyrosine, three valines, serine, and dec-3-enoic acid. The Adpa unit showed no three-bond COSY correlations between H-28 and H-29, and 1D and 2D TOCSY also indicated discontinuity of this spin system. HMBC correlations from H-27 to C-29, however, allowed the assignment of this unit. ROESY correlations between H-28 and H-29 also supported this partial structure. The geometry of the double bond (C-17, C-18) in Ahpea was assigned as *E* by the ^{1}H coupling constant (15.5 Hz) between H-17 and H-18 measured in DMSO-d_6 (these proton signals are second order in pyridine-d_5). The double bond geometry at C-41 and C-42 was established as *Z* also by the 11.0 Hz coupling constant observed between H-41 and H-42. This geometry was confirmed by a strong ROESY cross-peak between these protons. The sequence of the amino acid residues was readily determined by analysis of HMBC and ROESY spectral data.

The absolute configurations of the amino acids in **1** were assigned by application of the advanced Marfey method.[6] Prior to acid hydrolysis, the double bonds in Ahpea and Dea were hydrogenated to enhance the acid stability of the Ahpea unit by converting it to 4-amino-5-hydroxy-pentanoic acid (Ahpa). Acid hydrolysis of the hydrogenated product of **1** yielded the expected free amino acids, which were derivatized using L- and D-FDLA (advanced Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-leucine amide) and analyzed by LC/MS.

The absolute configurations of α-amino acid residues, assigned as L, were established in a straightforward fashion by LC/MS analysis. The determination of the absolute configuration at C-19 in Ahpa required an additional experi-

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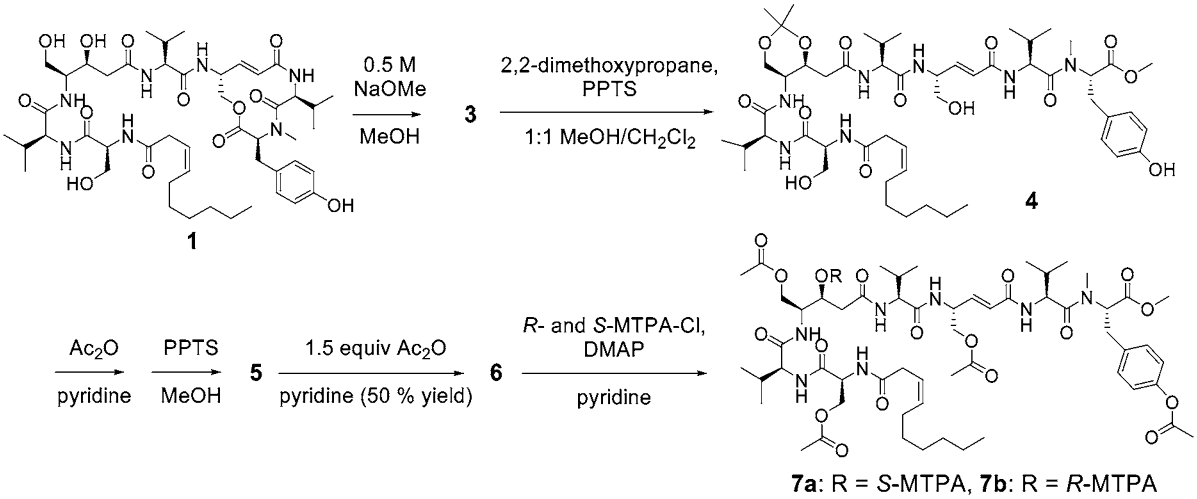
(6) (a) Fujii, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 3346. (b) Fujii, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 5146. (c) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.

Scheme 1. Reaction Sequence for Determination of the Absolute Configuration of 4-Amino-3,5-dihydroxy-pentanoic Acid



ment (see the Supporting Information). Initially, the L- and D-FDLA derivatives of Ahpa were detected by LC/MS analysis at 23.2 and 22.3 min, respectively. Because Ahpa is a γ-amino acid, which bears two additional methylenes, the elution sequence for the L- and D-FDLA derivatives is difficult to predict. This problem was solved by comparing the retention times of the FDLA derivatives of 4-amino-butyric acid and ethanolamine, which are the fragments of Ahpa. The FDLA derivative of 4-amino-butyric acid eluted later than that of ethanolamine, indicating that the acid chain of Ahpa is more hydrophobic than the serine side chain. This result allowed the prediction of the elution sequence of the L- and D-FDLA derivatives of Ahpa, the D-derivative eluting earlier than the L-derivative. On the basis of this result, the absolute configuration at the C-19 chiral center in Ahpa was established as *R*. Subsequently, the original amino acid Ahpea was assigned the 19*R* configuration.

This configuration at C-19 is also supported by transannular ROESY correlations in the 12-membered ring (Figure 1). The methyl group protons (H-10) of *N*-methyltyrosine showed clear ROESY correlations with H-17 and 19-NH, indicating this methyl group, H-17, and 19-NH are oriented into the ring. Another key correlation between H-12 and H-17 also defined the ring conformation as shown in Figure 1. These transannular ROESY correlations are not consistent with a possible 19*S* configuration.[7]

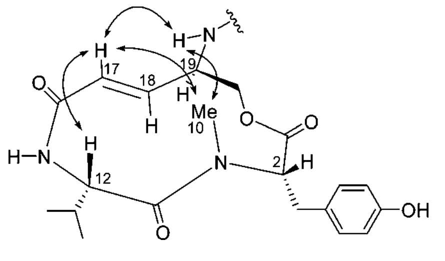


Figure 1. Transannular key ROESY correlations in the 12-membered ring in **1**.

To establish the relative configurations at C-28 and C-29 of Adpa, the methanolysis product of **1** (**3**) was converted to the acetonide **4** with 2,2-dimethoxypropane and pyridinium *p*-toluenesulfate in 1:1 methanol/dichloromethane (Scheme 1). The ^{13}C NMR shifts of the two methyl acetonide chemical shifts [δ_C: 19.5; 29.8] revealed that the methyl groups were in axial and equatorial positions in a six-membered 1,3-dioxane chair conformation.[8] Analysis of gCOSY and TOCSY spectral data allowed the assignment of the proton signals. In the ROESY NMR spectrum, the axial methyl group [δ_C: 19.5; δ_H: 1.36] clearly showed correlations with H-28 [δ_H: 4.22] and H-30a [δ_H: 4.09], indicating these protons are axial. Proton H-29 [δ_H: 3.68] displayed ROESY correlations with H-27a [δ_H: 2.33], H-27b [δ_H: 2.01], H-28 [δ_H: 4.22], H-30a [δ_H: 4.09], and H-30b [δ_H: 3.47], which established its equatorial position and indicated a 28*S**,29*S** assignment. A ROESY NMR cross-peak between 29-NH [δ_H: 7.85] and H-27a [δ_H: 2.33] also supported this assignment (Figure 2).

The absolute configuration of 4-amino-3,5-dihydroxy-pentanoic acid was determined by the modified Mosher method.[9] Deprotection of the acetonide after acetylation of **4** furnished the triacetate **5**. Timed acetylation of **5** allowed esterification mainly at the primary alcohol at C-30 to yield the tetraacetate **6**. The intact secondary hydroxyl group at C-28 in **6** was then treated with *R*-(−) and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenyl acetyl chloride (MTPA-Cl) to yield the *S*- and *R*-MTPA esters (**7a** and **7b**), respectively (Scheme 1). The proton chemical shifts of relevant protons were assigned by analysis of ^{1}H, ^{1}H−^{1}H gCOSY, and TOCSY NMR spectra. Calculation of $\Delta\delta_{S-R}$ values clearly defined

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[7] The MM2 energy-minimized structures by Chem3D for both 19*R* and 19*S* configurations are shown in the Supporting Information.

[8] Rychnovsky, S. D.; Rogers, B. N.; Richardson, T. I. *Acc. Chem. Res.* **1998**, *31*, 9.

[9] Séco, J. M.; Quiñoa, E.; Riguera, R. *Tetrahedron: Asymmetry* **2001**, *12*, 2915.

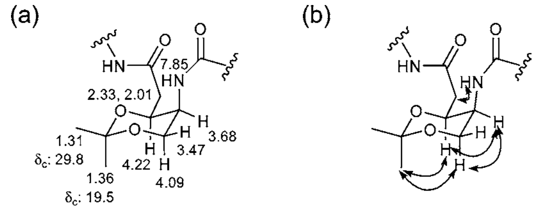

Figure 2. Chemical shifts (a) and key ROESY correlations (b) in the acetonide **4** of 4-amino-3,5-dihydroxy-pentanoic acid (Adpa).

the absolute configuration of C-28 as *S* and subsequently established the 29*S* configuration on the basis of their relative stereochemistry (see the Supporting Information).

Thalassospiramide B (**2**)[10] was obtained as an oil, which analyzed for the molecular formula $C_{56}H_{83}N_7O_{13}$ by ESI high-resolution mass spectral data. This molecular formula was also supported by ^{1}H and ^{13}C NMR data (see the Supporting Information), which displayed a high degree of similarity to thalassospiramide A (**1**). Further analysis of 1D and 2D NMR spectra allowed the assignment of *N*-methyltyrosine, three valine residues, Ahpea, Adpa, and Dea, but not serine as in thalassospiramide A. The difference in **2** was the replacement of a serine unit in **1** with 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa), another γ-amino acid. Long-range carbon−proton correlations in the HMBC NMR spectrum of **2** defined the same connectivity of the eight discrete spin systems as in **1**. The sequence of these subunits was identical to that of **1** except for the incorporation of Ahppa instead of serine.

The absolute configurations of the standard amino acid units were also determined using the advanced Marfey method, as in **1**. In this experiment, the *N*-methyltyrosine and three valines were assigned L configurations. The absolute stereochemistry of the Ahpea unit was established as 19*R* by transannular ROESY correlations indentical to those shown in Figure 1. The relative configurations (C-38 and C-39) of Ahppa were proposed by analysis of ^{1}H coupling constants and ROESY correlations. Specifically, no observable correlations in the gCOSY and TOCSY spectra were indicative of zero coupling between H-38 and H-39. This led us to establish the dihedral angle between these two protons at near 90° and allowed ROESY spectral analysis (Figure 3). Only the relative configurations 38*S** and 39*S** were in agreement with all observed ROESY correlations.[11]

Thalassospiramides A and B are the first secondary metabolites from the genus *Thalassospira*. These peptides are unique in that they incorporate unusual λ-amino acids such as 4-amino-5-hydroxy-penta-2-enoic acid (Ahpea), 4-amino-3,5-dihydroxy-pentanoic acid (Ahpa), and 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa). The rare Ahpea and Ahpa units were reported only once interestingly in peptides from the marine α-proteobacterium *Oceanospirillum* (strain SANK 70992).[12] The Ahppa unit was previously reported in compounds from the actinomycete *Streptomyces*[13] and the marine cyanobacterium *Symploca* sp.[10] The structural features of **1** and **2** are similar to those for compound B1371B from strain SANK 70992, with respect to the alternative combination of valines and λ-amino acids and the presence of the hydrocarbon chains.

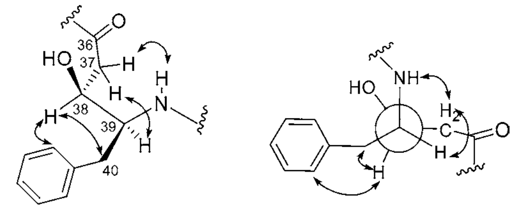

Figure 3. Key ROESY correlations in 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa).

The biological activities of **1** and **2** were evaluated in a mouse mixed lymphocyte assay, which probes for noncytotoxic suppression of cytokine production by ovalbumin (OVA) stimulated splenocytes. The thalassospiramides were initially screened for inhibition of IL-5 as a representative cytokine, which has been shown to play an important role in TH-2 mediated inflammatory diseases such as asthma.[14] In this assay, thalassospiramides A and B showed IC_{50} values of 10 and 5 μM with no observable cytotoxicity at concentrations of 10 μM. Further studies will be needed to assess in vivo activity and their molecular and cellular mechanisms of action before their utility can be accessed.

Acknowledgment. This work is a result of generous financial support provided by the NIH, National Cancer Institute, under grant CA44848. We thank Dr. Hak Cheol Kwon, Korea Institute of Science and Technology, for his generous help in the determination of stereochemistry of thalassospiramide A.

Supporting Information Available: NMR spectral data tables and complete 2D spectra of **1** and **2**, ^{1}H NMR of **3**−**8**, gCOSY, gHSQC, and ROESY spectra of **4**, the details of strain CNJ-328, mouse splenocyte assay, isolation of **1** and **2**, and chemical modifications of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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[10] Thalassospiramide B (**2**): oil; $[\alpha]_D$ −39 (*c* 0.39, CH_3CN); IR (neat) ν_{max} 3307, 2966, 1743, 1643, 1531 cm^{-1}; UV (CH_3CN) λ_{max} (log ϵ) 224 (4.2), 278 (3.4) nm; NMR spectral data, see Table S2 in the Supporting Information; HR-ESI-TOFMS $[M + Na]^+$ *m/z* 1084.5932 ($C_{56}H_{83}N_7O_{13}$-Na, calcd $[M + Na]^+$ 1084.5941).

[11] The consistent ROESY correlations were reported in the same unit in tasiamide B (marine cyanobacterium), of which absolute configurations were proposed as *S* and *S*. See: Williams, P. G.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2003**, *66*, 1006.

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[13] (a) Sato, T.; Shibazaki, M.; Yamaguchi, H.; Abe, K.; Matsumoto, H.; Shimizu, M. *J. Antibiot.* **1994**, *47*, 588. (b) Omura, S.; Imamura, N.; Kawakita, K.; Mori, Y.; Yamazaki, Y.; Masuma, R.; Takahashi, Y.; Tanaka, H.; Huang, L.-Y.; Woodruff, H. B. *J. Antibiot.* **1986**, *39*, 1079.

[14] Greenfeder, S.; Umland, S. P.; Cuss, F. M.; Chapman, R. W.; Egan, R. W. *Respir. Res.* **2001**, *2*, 71.