Bioorganic & Medicinal Chemistry Letters 20 (2010) 3658–3663

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Trichoderins, novel aminolipopeptides from a marine sponge-derived *Trichoderma* sp., are active against dormant mycobacteria

Patamaporn Pruksakorn [a,†], Masayoshi Arai [a,†], Naoyuki Kotoku [a], Catherine Vilchèze [b], Anthony D. Baughn [b], Prashini Moodley [c], William R. Jacobs Jr. [b], Motomasa Kobayashi [a,*]

[a] *Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan*
[b] *Howard Hughes Medical Institute, Price Translational Research Center, Albert Einstein College of Medicine, 1301 Morris Park Avenue, Bronx, NY 10461, USA*
[c] *Nelson R. Mandela School of Medicine, University of KwaZulu Natal, 719 Umbilo Road, Congella Durban 4013, South Africa*

ARTICLE INFO

Article history:
Received 2 March 2010
Revised 17 April 2010
Accepted 21 April 2010
Available online 26 April 2010

Keywords:
Trichoderin
Trichoderma sp.
Aminolipopeptide
Anti-mycobacterial activity
Dormant
Tuberculosis

ABSTRACT

Three new aminolipopeptides, designated trichoderins A (**1**), A1 (**2**), and B (**3**), were isolated from a culture of marine sponge-derived fungus of *Trichoderma* sp. as anti-mycobacterial substances with activity against active and dormant bacilli. The chemical structures of trichoderins were determined on the basis of spectroscopic study. Trichoderins showed potent anti-mycobacterial activity against *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, and *Mycobacterium tuberculosis* H37Rv under standard aerobic growth conditions as well as dormancy-inducing hypoxic conditions, with MIC values in the range of 0.02–2.0 μg/mL.

Tuberculosis is one of the most common causes of morbidity and mortality in HIV-positive adults living in poverty.[1] Aggravation of tuberculosis is evaded by host immune systems, even though infection is concluded completely. However, a small population of bacilli enter into a dormant state in the granuloma which is formed by immune cells and fibroblasts in an attempt to contain the infection. Then, the bacilli maintain their ability to resume growth and aggravate disease as a result of deterioration of immune system. This unique property also relates to tolerance to conventional anti-tuberculosis drugs such as isoniazid.[2] Therefore, new lead compounds, which are effective against *Mycobacterium tuberculosis* in both active and dormant states, are urgently needed. Although physiology of the latent *M. tuberculosis* infection is still unclear, hypoxic incubation was found to induce the dormant state of *Mycobacterium* sp., which has a drug susceptibility profile resembling that of the latent *M. tuberculosis*.[3]

Recently, we established a screening system to search for substances that have activity against dormant mycobateria, and isolated macrocyclic alkaloids, halicyclamines, from a marine sponge of *Haliclona* sp. on the basis of bioassay-guided separation.[4] In the continuous screening from marine organisms, we isolated three new aminolipopeptides named trichoderins A (**1**),[5] A1 (**2**),[6] and B (**3**)[7] from a culture of marine sponge-derived fungus of *Trichoderma* sp. 05FI48 as anti-dormant mycobacterial substances. In this paper, the structure elucidation of trichoderins and their anti-mycobacterial activities are presented.

The fungal strain 05FI48 of *Trichoderma* sp. was isolated from unidentified marine sponge in 2005. The strain was cultured in the rice medium containing artificial sea water (totally 2.3 kg of unpolished rice and 4.5 L of artificial sea water) under static condition at 30 °C for 2 weeks. The culture was extracted by acetone and mixed organic solvent of acetone/methanol/ethyl acetate (4:2:1), and the crude extract was partitioned into a water/2-butanone mixture. The 2-butanone soluble portion (51.2 g) was further partitioned with an *n*-hexane and 90% methanol mixture. On the guidance of bioassay, the 90% methanol soluble portion (37.7 g, MIC 12.5 μg/mL against *Mycobacterium bovis* BCG under hypoxic condition) was fractionated by silica gel column chromatography (eluted ethyl acetate/methanol) to give eight fractions (Fr. A1-Fr. A8). The active Fr. A4 (ethyl acetate/methanol = 5:1 eluted) was dissolved in methanol. Then, the MeOH soluble portion (2.5 g, MIC 2.5 μg/mL) was fractionated by silica gel column chromatography (chloroform/methanol/water = 30:3:1, lower phase) to afford four fractions (Fr. B1-Fr. B4). The active Fr. B2 (390 mg, MIC 0.15 μg/mL) was subjected to Sephadex LH-20 column chromatography and eluted with methanol. The active fraction (243 mg, MIC

* Corresponding author. Tel.: +81 66879 8215; fax: +81 66879 8219.
E-mail address: kobayasi@phs.osaka-u.ac.jp (M. Kobayashi).
† Both authors contributed equally to this work.

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doi:10.1016/j.bmcl.2010.04.100

0.04 μg/mL) was purified by reversed-phase HPLC [CAPCELL PAK C_8 DD (10 mm id × 250 mm); eluted with acetonitrile/water containing 0.1% TFA = 7:3] to furnish trichoderin A (**1**) (89 mg, 0.174% yield from the 2-butanone extract), trichoderin A1 (**2**) (18 mg, 0.035%), and trichoderin B (**3**) (8 mg, 0.016%) as TFA salts, respectively (Fig. 1).

Trichoderin A (**1**) was obtained as a colorless amorphous solid. The ESI-TOF MS of **1** showed a pseudomolecular ion peak at *m/z* 1163 $[M]^+$. The molecular formula was determined as $C_{60}H_{111}N_{10}O_{12}$ by high-resolution (HR-) ESI-TOF MS. The IR absorption at 3314 $cm^{-1}$and 1669 cm^{-1} suggested the presence of hydroxyl and/or amide groups, respectively. The observation of the signals at 175–178 ppm in the ^{13}C NMR spectrum and the downfield signals of the ^1H NMR spectrum indicated peptide structure of **1**. The upfield signals of the ^1H and ^{13}C NMR spectra implied the presence of aliphatic hydrocarbons in **1**. These finding suggested that compound **1** is an aminolipopeptide.

To confirm the amino acid composition of **1**, compound **1** was treated by dabsyl chloride following to acid hydrolysis.[8] HPLC analysis of the dabsylated amino acids revealed that compound **1** contained Pro, Aib (α-aminoisobutyric acid), Ile and Val in the structure. The order of amino acid sequence was determined through the observation of a series of the fragment ions by ESI-TOF-MSMS (Table 1 and Fig. 2). The fragment ions from ESI-TOF-MSMS experiments indicated cleavage of the amide bond leading the predominant B-type fragmentation.[9] In addition, the difference of 213 amu. as shown in Figure 2 suggested the presence of an unusual amino acid, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD), from the reported data of MSMS analysis in the structure-related aminolipopeptides, leucinostatins,[10] trichopolyns,[11] helioferins,[12] and roseoferins.[13]

The chemical structure of **1** was also confirmed by NMR analysis. The ^{13}C NMR, DEPT, and HMQC spectra of **1** showed 60 carbon-signals that were classified as 18 methyls, 18 methylenes, 10 methines, four quaternary carbons, nine amide carbonyls and one ketone carbonyl. Since the last step for purification of **1** was carried out under acidic condition, **1** was isolated as the protonated form of the amino group in the C-terminal residue as observing of the

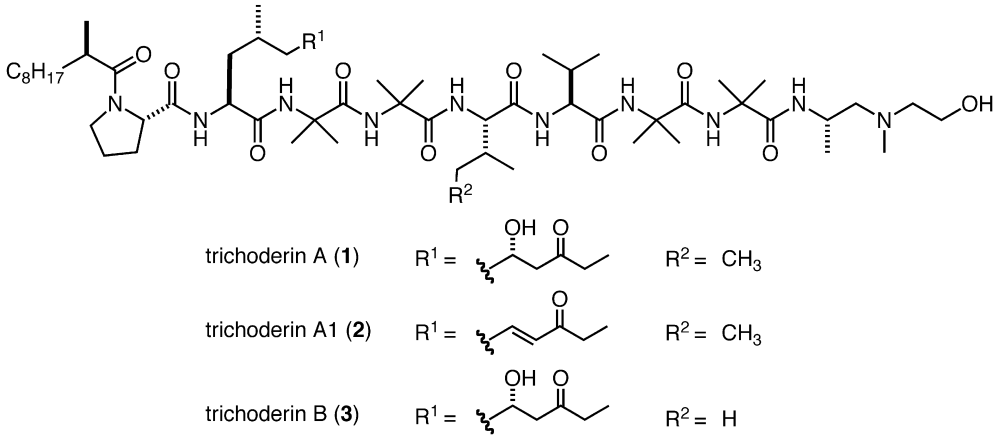
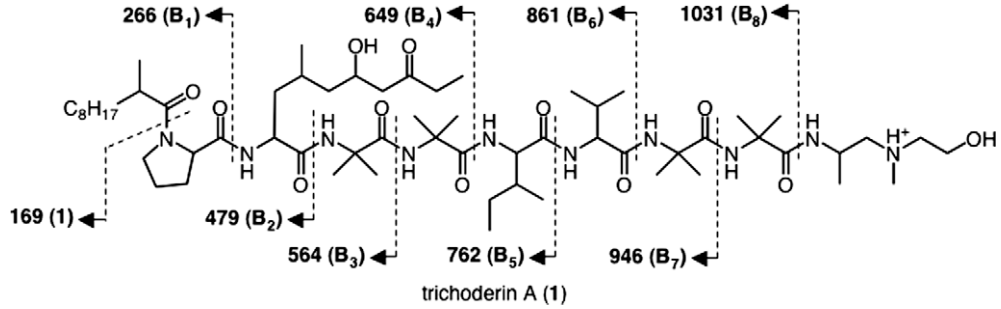


Figure 1. Chemical structures of trichoderins (**1**–**3**).

Table 1
ESI-TOF-MSMS analysis of trichoderins A (**1**), A1 (**2**) and B (**3**)



	1	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8	$[M]^+$
1	169	266	479	564	649	762	861	946	1031	1163
2	169	266	**461**	546	631	744	843	928	1013	1145
3	169	266	479	564	649	**748**	847	932	1017	1149
1	MDA	Pro	AHMOD	Aib 1	Aib 2	Ile	Val 2	Aib 3	Aib 4	AMAE
2	MDA	Pro	**AMOD**	Aib 1	Aib 2	Ile	Val 2	Aib 3	Aib 4	AMAE
3	MDA	Pro	AHMOD	Aib 1	Aib 2	**Val 1**	Val 2	Aib 3	Aib 4	AMAE

MDA; 2-methyl decanoic acid. Pro; proline. AHMOD; 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid, AMOD; 2-amino-4-methy1-8-oxodec-6-enoic acid, Aib; α-amino-isobutyric acid, Ile; isoleucine, Val; valine. AMAE; 2-[(2′-aminopropyl) methylamino] ethanol.

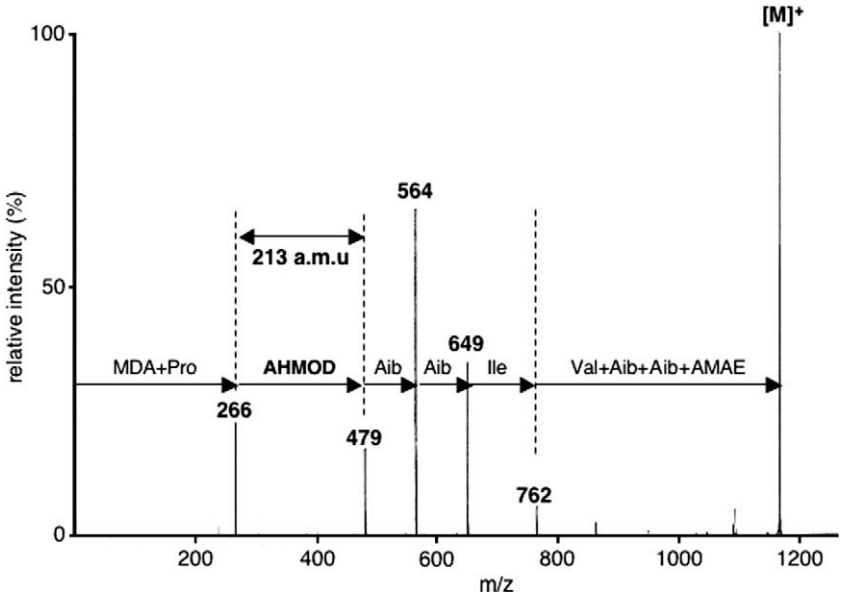


Figure 2. ESI-TOF-MSMS spectra for **1**.

proton signal at δ_H 8.23. The presence of this quaternary amine clarified the connection in the C-terminal residue by ^1H-^1H connectivities ($^2J_{(H,H)}$ coupling) of the quaternary amino proton at δ_H 8.23 and the adjacent protons at δ_H 3.06 (*N*-CH_3), δ_H 3.09 (H-1′) and δ_H 2.92 (H-2). In addition, the heteronuclear long range coupling ($^3J_{(C,H)}$) between the *N*-CH_3 at δ_H 3.06 and the methylene carbons at δ_C 54.5 (C-2) and δ_C 62.4 (C-1′) completely proved the connection of this part (Fig. 3 and Table 2). Thus, the C-terminus of **1** was deduced to be 2-[(2′-aminopropyl) methylamino] ethanol (AMAE).

The existing of AHMOD was confirmed by COSY and HMBC analysis as the correlations between the ketone carbonyl at δ_C 212.3 (C-8) and the two adjacent methylene protons at δ_H 2.52 (H-9) and δ_H 2.59, 2.43 (H-7). (Fig. 3 and Table 2)

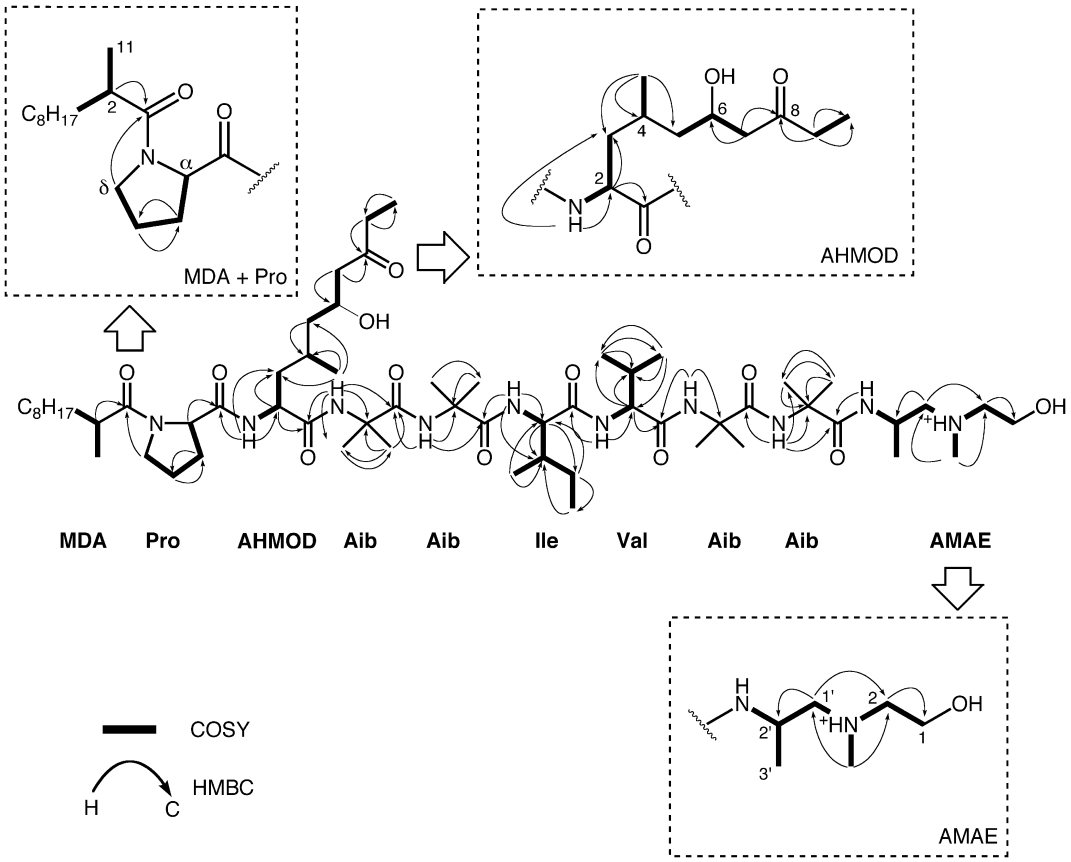


Figure 3. COSY and HMBC correlations for **1**.

Table 2
^{1}H and ^{13}C NMR data for trichoderins A (**1**), A1 (**2**) and B (**3**)

Position		1		2		3	
		δ_C[a]	δ_H[b,c]	δ_C[a]	δ_H[b,c]	δ_C[a]	δ_H[b,c]
MDA[d]	1	177.1		177.4		177.9	
	2	38.0	2.56	37.9	2.65	38.3	2.81
	3	34.2	1.25, 1.49	34.2	1.28, 1.50	34.2	1.34, 1.50
	4	27.3	1.20	27.2	1.23	27.4	1.28
	5	29.5	1.21	29.4	1.21	29.5	1.25
	6	29.9	1.25	29.9	1.25	30.0	1.27
	7	29.9	1.25	29.7	1.25	30.0	1.27
	8	32.1	1.24	32.1	1.24	32.1	1.24
	9	29.7	1.23	29.6	1.24	29.7	1.26
	10	14.3	0.87	14.3	0.88	14.3	0.87
	11	15.7	1.12 (d, 6.4)	15.9	1.12 (d, 6.3)	16.4	1.21
Pro	CO	175.2		175.1		174.6	
	α	62.7	4.17	62.7	4.18	62.7	4.18
	β	30.4	1.96, 2.16	29.9	1.99, 2.24	30.4	1.97, 2.17
	γ	25.3	1.92, 2.16	25.3	1.92, 2.23	25.3	1.94, 2.14
	δ	47.6	3.44, 3.99	47.6	3.48, 4.02	47.8	3.48, 4.18
AHMOD/NH			9.07 (br)		9.13 (br)		9.01 (br)
AMOD	1	175.5		175.3		175.1	
	2	55.4	4.14	55.4	4.12	55.0	4.14
	3	34.7	1.25, 2.16	36.7	1.62, 1.83	35.8	1.95
	4	25.9	1.99	28.7	2.23	25.7	2.14
	5	45.3	1.26, 1.46	39.6	1.99, 2.34	45.5	1.24, 1.50
	6	64.5	4.06	145.8	6.75	64.1	4.23
	7	50.9	2.43, 2.59	131.9	6.02 (d, 15.7)	50.8	2.41, 2.61
	8	212.3		201.7		212.5	
	9	37.0	2.52	33.0	2.55 (q, 7.2)	37.1	2.54
	10	7.7	1.03	8.4	1.07 (t, 7.2)	7.8	1.01
	11	20.2	0.88	19.2	0.85	20.0	0.85
Aib 1	CO	176.6		176.4		175.9	
	α	56.5		56.3		56.7	
	β	22.6	1.38	22.4	1.42	22.6	1.48
		26.8	1.49	26.7	1.51	26.9	1.54
Aib 2	CO	177.3		177.2		177.2	
	α	56.4		56.4		56.5	
	β	22.1	1.41	22.1	1.39	22.6	1.42
		27.2	1.51	27.3	1.52	27.1	1.52
Ile/Val 1	NH		7.58 (d, 5.6)		7.64 (d, 5.5)		7.48 (d, 5.6)
	CO	175.3		175.2		175.0	
	α	62.1	3.63	62.0	3.68	63.5	3.58
	β	35.8	1.99	35.8	1.99	29.5	2.21
	γ	27.1	1.23, 1.82	26.7	1.24, 1.50	20.5	1.13 (d, 6.7)
	δ	15.6	0.94 (d, 6.7)	15.6	0.96 (d, 6.7)	19.5	0.99 (d, 7.0)
		11.5	0.88	11.4	0.88		
Val 2	NH		7.93		7.96 (d, 4.9)		7.83 (d, 5.6)
	CO	175.7		175.8		175.5	
	α	65.5	3.36 (dd, 11.4, 5.3)	65.5	3.37 (dd, 11.6, 5.5)	65.2	3.38
	β	28.7	2.22	30.2	2.23	28.8	2.20
	γ	21.2	1.02	21.2	1.01 (d, 6.3)	21.1	1.02
		19.2	0.87	18.9	0.88	19.2	0.87
Aib 3	CO	177.4		177.3		177.0	
	α	56.8		56.8		57.0	
	β	22.9	1.41	22.9	1.41	22.9	1.41
		27.4	1.52	27.4	1.54	27.4	1.52
Aib 4	CO	175.9		176.0		176.5	
	α	57.1		57.1		56.6	
	β	22.5	1.49	22.5	1.42	22.9	1.38
		26.6	1.54	27.1	1.52	27.1	1.50
AMAE	NH (2′-aminopropyl)	7.71 (d, 10.0)		7.74 (d, 9.6)		7.69 (d, 10.0)	
	1	56.3	3.88, 4.11	56.2	3.87, 4.11	56.3	3.90, 4.12
	2	54.5	2.92, 3.77	54.4	2.84, 3.80	54.2	2.92, 3.81
	1′	62.4	3.09, 3.58	62.5	3.04, 3.60	62.5	3.17, 3.58
	2′	39.8	4.60	39.8	4.61	40.0	4.62
	3′	18.1	1.25	18.1	1.24	18.0	1.24
	N-CH_3	42.4	3.06 (d, 4.4)	42.3	3.05 (d, 4.2)	42.5	3.08 (d, 5.0)
	NH (quaternary amine)		8.23 (br)		8.25 (br)		8.24 (br)

[a] ^{13}C NMR: δ_C (ppm), (150 MHz, $CDCl_3$).
[b] ^{1}H NMR: δ_H (ppm, *J* in Hz), (600 MHz, $CDCl_3$).
[c] Multiplicity was not reported because of overlapping resonances.

The observation of heteronuclear long range coupling between the amide carbonyl at δ_C 177.1 (C-1) and the δ-protons of proline at δ_H 3.99, 3.44 confirmed that the N-terminus of **1** was associated with 2-methyl decanoic acid (MDA) (Fig. 3 and Table 2). Moreover, the strong NOE correlation between the α-methyl protons of MDA (δ_H 1.12) and the δ-proton of proline (δ_H 3.99) also completely proved the connection of N-terminal residue (Fig. 4). All the proton- and carbon-signals were assigned as shown in Table 2.

Stereostructure of chiral amino acids were deduced by HPLC analysis of the derivatized amino acids by 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent).[14] The result of HPLC analysis indicated that all the constituting chiral amino acid of **1** were found to be L-amino acids. Stereostructure of N-terminal, AHMOD and C-terminal were tentatively assigned based on the NOE correlations and the comparison with the structurally related compounds, trichopolyns.[11] Thus, the N-terminal region was presumed to take a conformation where the NOE correlations [between the pro-*R* δ-proton of proline (δ_H 3.99) and the α-proton of proline (δ_H 4.17) and the α-methyl protons of MDA (δ_H 1.12); the β-proton of MDA (H-3, δ_H 1.25) and the pro-*S* δ-proton of proline (δ_H 3.44)] were observed as shown in Figure 4. The observation of the NOE correlations [between the α-proton (H-2, δ_H 4.14) and the γ-methyl protons of AHMOD (δ_H 0.88); the H-5 proton (δ_H 1.26) and the adjacent protons (H-4 proton (δ_H 1.99) and the H-6 proton (δ_H 4.06)); the H-3 proton (δ_H 2.16) and the H-6 proton] suggested that AHMOD might take a conformation as shown in Figure 4. Furthermore, the NOE correlations [between the 2′-proton (H-2′, δ_H 4.60) and the adjacent proton (H-1′, δ_H 3.09), the 2-methylene protons (H-2, δ_H 2.92 and 3.77); the 1′-proton (H-1′, δ_H 3.58) and the *N*-methyl protons of AMAE (δ_H 3.06)] revealed a conformation of AMAE residue as shown in Figure 4. From these evidences and the comparison with stereochemistry of trichopolyn I (**4**), the relative stereostructure of **1** was tentatively assigned as depicted in Figure 4.

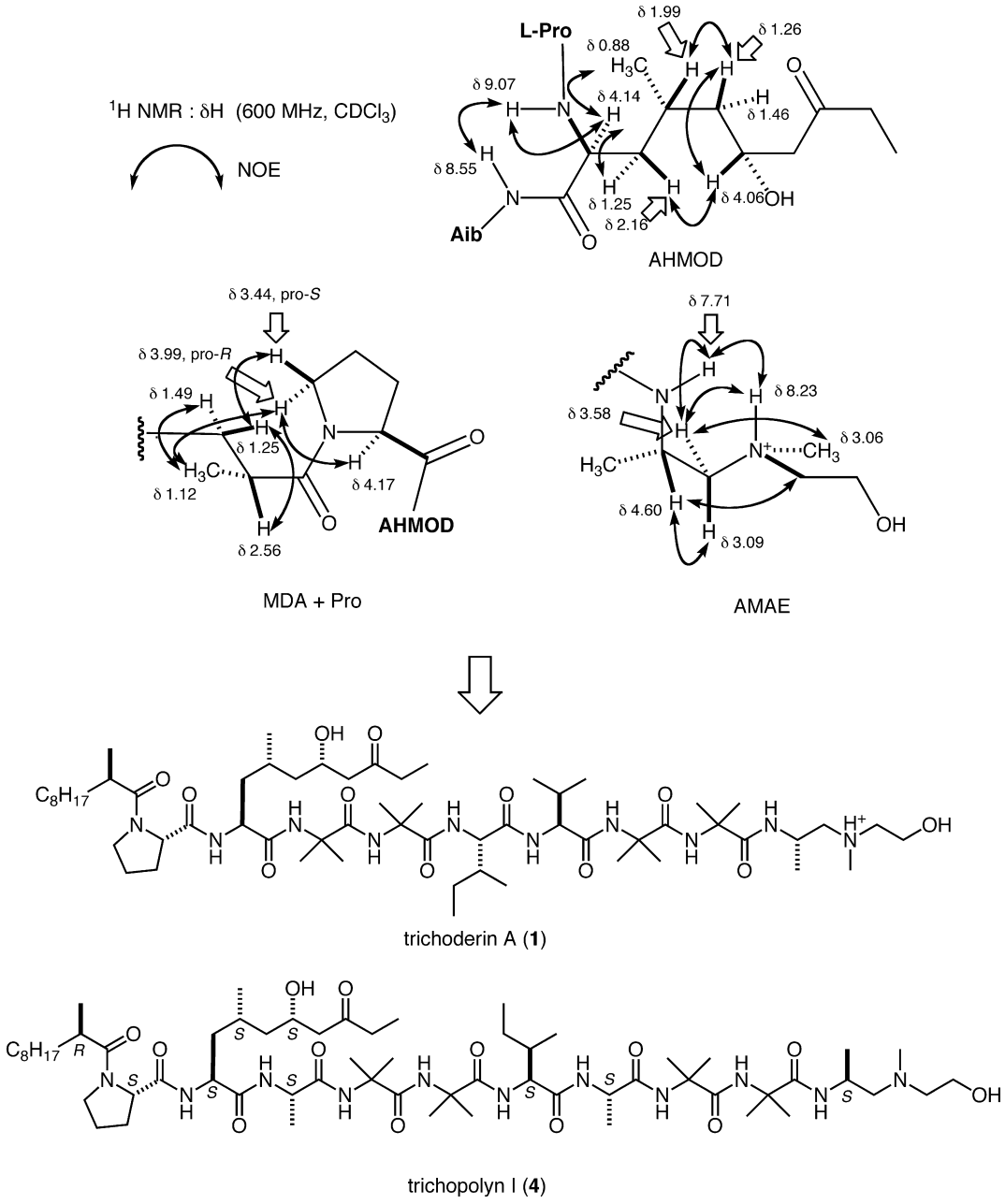


Figure 4. NOE correlations and plausible stereochemistry of **1**.

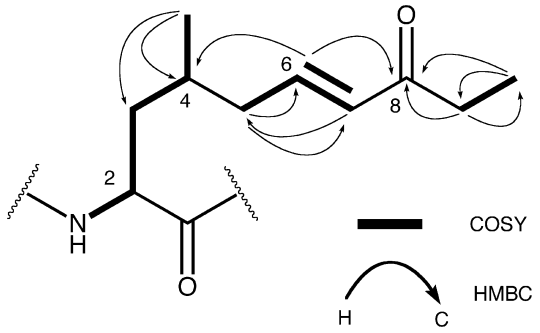


Figure 5. COSY and HMBC correlations for AMOD moiety in **2**.

Table 3
MIC values of trichoderins (**1**–**3**) and isoniazid against *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Rv under both aerobic and hypoxic conditions

Compounds	MIC (μg/mL)					
	M. smegmatis		*M. bovis* BCG		*M. tuberculosis* H37Rv	
	Aerobic	Hypoxic	Aerobic	Hypoxic	Aerobic	Hypoxic
1	0.1	0.1	0.02	0.02	0.12	0.12
2	1.56	1.56	0.16	0.16	2.0	2.0
3	0.63	0.63	0.02	0.02	0.13	0.13
Isoniazid	2.5	25	0.03	>100	0.05	>100

Trichoderin A1 (**2**) was obtained as a colorless amorphous solid. The ESI-TOF-MS of **2** showed a pseudomolecular ion peak at *m/z* 1145 $[M]^+$, which was smaller than that of **1** by 18 mass units, and the molecular formula was determined as $C_{60}H_{109}N_{10}O_{11}$ by HR-ESI-TOF-MS. The comparison of the fragmentation patterns between **1** and **2** by ESI-TOF-MSMS analysis suggested that compound **2** has AMOD moiety instead of AHMOD as shown in Table 1. This moiety was confirmed by the heteronuclear long range coupling ($^3J_{(C,H)}$) between the olefinic proton at H-6 (δ_H 6.75) and the ketone carbonyl at C-8 (δ_C 201.7) (Fig. 5). All the proton- and carbon-signals were assigned as shown in Table 2.

Trichoderin B (**3**) was obtained as a colorless amorphous solid. The ESI-TOF-MS of **3** showed a pseudomolecular ion peak at *m/z* 1149 $[M]^+$, and the molecular formula was determined as $C_{59}H_{109}N_{10}O_{12}$ by HR-ESI-TOF-MS. The ESI-TOF-MSMS analysis of **3** confirmed that the B_5 fragment of **3** was assigned to Val instead of Ile in **1** (Table 1). The presence of valine in **3** was also supported by the detailed analysis of 2D NMR spectra. All the proton- and carbon-signals were assigned as shown in Table 2.

MIC values of trichoderins (**1**–**3**) and isoniazid against *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Rv under the both aerobic condition and hypoxic condition of nitrogen atmosphere containing 0.2% oxygen inducing dormant state were determined by the established method previously.[4] The dormant *M. tuberculosis* was highly resistant against isoniazid, which inhibits inhA of the type II fatty acid synthase.[3a] As shown in Table 3, the MIC values of isoniazid against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv were 2.5 μg/mL, 0.03 μg/mL, and 0.05 μg/mL under aerobic condition, respectively. While, the MIC values of isoniazid against these strains were more than 25 μg/mL under nitrogen atmosphere containing 0.2% oxygen. On the other hand, trichoderins (**1**–**3**) showed potent anti-mycobacterial activity against *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Rv under both aerobic conditions and dormancy-inducing hypoxic conditions, with MIC values in the range of 0.02–2.0 μg/mL. These results indicated that trichoderins were effective against *Mycobacterium* sp. in both actively growing and dormant states. Among them, **1** and **3** having AHMOD moiety in the structures showed the more potent anti-mycobacterial activity against the pathogenic strain *M. tuberculosis* H37Rv with MIC values of 0.12 and 0.13 μg/mL, respectively. This observation suggested that the AHMOD moiety is important for anti-mycobacterial activity of trichoderins. Action-mechanism of trichoderins is currently under study.

Acknowledgments

The authors thank Ms. Salona Moodley, Mr. Yushir R. Maharaj, Ms. Karnishree Govender (University of KwaZulu Natal) for excellent technical assistance. A.D.B. was supported by a Grant from the Helen Hay Whitney Foundation. This study was financially supported by the Hoh-ansha Foundation, the Uehara Memorial Foundation, JSPS-South Africa (NRF) Joint Research Program, and Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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5. *Trichoderin A* (**1**): colorless amorphous solid. $[\alpha]_D^{20}$ −17 (*c* 0.7, MeOH). IR ν_{max} (KBr) cm^{-1}: 3314, 2928, 1669, 1535, 1200. ESI-MS: *m/z* 1163 $[M]^+$. High resolution ESI-MS: calcd for $C_{60}H_{111}N_{10}O_{12}$: *m/z* 1163.8383. Found 1163.8851. ^1H NMR (600 MHz, $CDCl_3$, δ_H), ^{13}C NMR (150 MHz, $CDCl_3$, δ_C) spectra: as shown in Table 2.
6. *Trichoderin A1* (**2**): colorless amorphous solid. $[\alpha]_D^{20}$ −23 (*c* 0.3, MeOH). IR ν_{max} (KBr) cm^{-1}: 3322, 2932, 2361, 1667, 1534, 1200. UV λ_{max} (MeOH) nm (ε): 232 (sh) (14100). ESI-MS: *m/z* 1145 $[M]^+$. High resolution ESI-MS: calcd for $C_{60}H_{109}N_{10}O_{11}$: *m/z* 1145.8277. Found 1145.8325. ^1H NMR (600 MHz, $CDCl_3$, δ_H), ^{13}C NMR (150 MHz, $CDCl_3$, δ_C) spectra: as shown in Table 2.
7. *Trichoderin B* (**3**): colorless amorphous solid. $[\alpha]_D^{20}$ −59 (*c* 0.1, MeOH). IR ν_{max} (KBr) cm^{-1}: 3318, 2930, 1667, 1535, 1200. ESI-MS: *m/z* 1149 $[M]^+$. High resolution ESI-MS: calcd for $C_{59}H_{109}N_{10}O_{12}$: *m/z* 1149.8226. Found 1149.8474. ^1H NMR (600 MHz, $CDCl_3$, δ_H), ^{13}C NMR (150 MHz, $CDCl_3$, δ_C) spectra: as shown in Table 2.
8. Trichoderins (5 mg each) were treated with 5 mL of 6 N HCl aq, and heated at 110 °C for 20 h. The reaction mixture was partitioned with ether. The aqueous portion was dried under reduced pressure to obtain crude amino acids. The crude amino acids from each trichoderins and authentic amino acids were dissolved in 40 μL of 50 mM sodium bicarbonate (pH 8.1) in 3 mL screw-cap glass tubes, respectively. Then, 80 μL of freshly prepared 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) solution (4 mM in acetonitrile) was added to each sample. The samples were sealed with caps and parafilm. The samples were then heated at 70 °C for 10 min. After dabsylation, the samples were analyzed by reversed-phase HPLC under the following condition; Cosmosil $5C_{18}$ AR, (4.6 mm id × 250 mm), a 100 min linear gradient from acetonitrile/25 mM sodium acetate buffer (pH 6.5) = 15:85–70:30, 37 °C, 1 mL/min, and detection at 436 nm. Authentic dabsylated Pro, Val, Aib and Ile were eluted with retention times of 44, 46, 48 and 51 min, respectively.
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14. The crude amino acids from each trichoderins and L- and D-authentic amino acids were dissolved in 100 μL of 0.5 M sodium bicarbonate in 3 mL screw-cap glass tubes, respectively. Then, 200 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDLA, Marfey's reagent, 10 mg/mL in acetone) was added to each sample. The samples were sealed with caps and incubated at 40 °C for 90 min. After addition of 25 μL of 2 M hydrochloric acid, the reaction mixture was diluted with methanol to suitable volumes (15–20-fold dilution). An aliquot of the L-FDLA derivatives was analyzed by reversed-phase HPLC under the following condition; Cosmosil $5C_{18}$ AR, (4.6 mm id × 250 mm), a 60 min linear gradient from acetonitrile/50 mM triethylamine phosphate (TEAP) buffer (pH 3.0) = 1:9–1:1, 1.5 mL/min, and detection at 340 nm. Authentic derivatized L-Pro, D-Pro, L-Val, L-Ile, D-Val and D-Ile were eluted with retention times of 26, 28, 34, 40, 41 and 45 min, respectively.