Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 191–194
Bioorganic & Medicinal Chemistry Letters

Himeic acid A: a new ubiquitin-activating enzyme inhibitor isolated from a marine-derived fungus, *Aspergillus* sp.

Sachiko Tsukamoto,[a,*] Hiroshi Hirota,[b,c] Misako Imachi,[d] Masahiro Fujimuro,[e] Hiroyuki Onuki,[b,c] Tomihisa Ohta[a] and Hideyoshi Yokosawa[e]

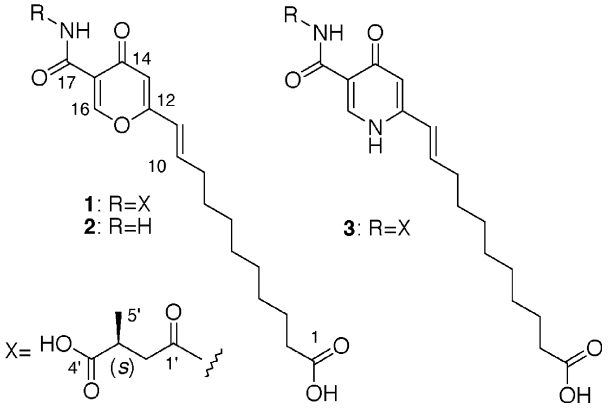
[a]*Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-1192, Japan*
[b]*RIKEN Genomic Sciences Center (GSC), 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan*
[c]*Graduate School of Integrated Sciences, Yokohama City University, 1-7-29 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan*
[d]*Bruker BioSpin K.K., 21-5, Ninomiya-3-chome, Tsukuba 305-0051, Japan*
[e]*Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*

Received 26 August 2004; revised 4 October 2004; accepted 5 October 2004
Available online 26 October 2004

Abstract—A new ubiquitin-activating enzyme (E1) inhibitor, himeic acid A, was isolated from a culture of marine-derived fungus, *Aspergillus* sp. The structure was determined by spectroscopic analysis. The formation of an E1–ubiquitin (Ub) intermediate was 65% inhibited by himeic acid A at the concentration of 50 μM, while two new related compounds, himeic acids B and C, showed little inhibitory activity even at 100 μM.

Ubiquitination of proteins requires the sequential action of three enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3).[1–4] Since E1 activity is essential for the ubiquitin–proteasome pathway that plays important roles in various cellular events, including cell cycle progression, transcriptional regulation, and signal transduction,[1–4] inhibitors of E1 could be useful for treatment of diseases related to the ubiquitin–proteasome pathway. In fact, several proteasome inhibitors show anti-cancer activity against various cancer cells that are resistant to conventional chemotherapeutic agents.[5] In the course of our search for inhibitors against the ubiquitin–proteasome pathway, we have already succeeded in isolating agosterol derivatives as proteasome inhibitors.[6] In addition, we found that girolline, an anti-cancer compound, is the first agent inhibiting the recruitment of polyubiquitinated p53 to the proteasome.[7] In this paper, we describe the isolation and structure elucidation of new compounds, himeic acids A–C (**1**–**3**), and their inhibitory activities against E1.

The fungus, *Aspergillus* sp.,[8] was separated from the mussel, *Mytilus edulis*, collected in Toyama Bay in the Japan Sea. The mycelium grown in the culture[9] (1.2 L) was extracted with MeOH. The EtOAc soluble part of the extract was partitioned between hexane and 90% MeOH–H_2O, and the latter fraction was purified by ODS chromatography and ODS HPLC to afford himeic acids A (**1**, 0.36 g),[10] B (**2**, 49.0 g),[11] and C (**3**, 18.4 mg).[12]



Keywords: Ubiquitin-activating enzyme inhibitor; Marine-derived fungus; *Aspergillus* sp.
* Corresponding author. Tel.: +81 76 234 4469; fax: +81 76 264 6241; e-mail: sachiko@p.kanazawa-u.ac.jp

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.10.012

Table 1. NMR spectral data for **1** in $CDCl_3$–C_5D_5N (1:1)

	δ_H (mult., Hz)		δ_C	HMBC (#C)
1			175.7 C	
2	2.40 (2H)	t, 7.3	34.2 CH_2	1, 3
3	1.72 (2H)	Quintet, 7.3	24.9 CH_2	1
4	1.38 (2H)	m	28.90[a] CH_2	3
5	1.29 (2H)	m	28.7[a] CH_2	
6	1.29 (2H)	m	28.83[a] CH_2	
7	1.29 (2H)	m	28.86[a] CH_2	
8	1.42 (2H)	m	27.9 CH_2	9, 10
9	2.19 (2H)	dt, 7.0, 7.3	32.5 CH_2	8, 10, 11
10	6.70	dt, 16.0, 7.0	142.8 CH	8, 9, 11, 12
11	6.07	d, 16.0	120.5 CH	9, 10, 12, 13
12			162.6 C	
13	6.32	s	113.2 CH	11, 12, 14, 15
14			177.58[b] C	
15			118.0 C	
16	8.76	s	162.2 CH	12, 14, 15, 17
17			161.0 C	
1′			172.8 C	
2′	2.99	dd, 17.7, 5.2	41.9 CH_2	1′, 3′, 4′, 5′
	3.52	dd, 17.7, 8.6		1′, 3′, 4′, 5′
3′	3.23	ddq, 8.6, 5.2, 6.4	35.0 CH	1′, 2′, 4′, 5′
4′			177.61[b] C	
5′	1.37 (3H)	d, 6.4	17.1 CH_3	2′, 3′, 4′
NH	12.04	s		15, 17, 2′

[a] May be interchangeable.
[b] May be interchangeable.

Himeic acid A (**1**) had a molecular formula of $C_{22}H_{29}NO_8$ as determined by HRFABMS, requiring nine degrees of unsaturation. Analysis on the basis of the ^1H and ^{13}C NMR data (Table 1) combined with the HMQC spectrum in $CDCl_3$–C_5D_5N (1:1) showed the presence of an aliphatic doublet methyl, a 1,2-disubstituted *trans*-olefin (δ_H 6.07; δ_C 120.5 and δ_H 6.70; δ_C 142.8), two singlet olefins (δ_H 6.32; δ_C 113.2 and δ_H 8.76; δ_C 162.2), a D_2O-exchangeable proton (δ 12.04), and seven quaternary carbons including three carbonyl carbons. Interpretation of the COSY and HMBC spectra allowed the assignment of two units, a 3-substituted dienone attached to a linear fatty acid C-1–C-14 (unit A) and a butanone substituted with a carbonyl carbon at the β-position C-1′–C-5′ (unit B). HMBC correlations between the two olefinic protons at δ_H 6.32 and 8.76 and three quaternary carbons δ_C 118.0, 162.6, and 177.58 indicated that the signals were accommodated on a 2,5-disubstituted 4-pyrone ring in which unit A was partially incorporated (Fig. 1). The D_2O-exchangeable proton showed HMBC correlation with two quaternary carbons at δ 118.0 (C-15) and δ 161.0 (C-17) and a methylene at δ 41.9 (C-2′), indicating that unit B was connected to the pyrone ring through an amide group. Connection of these units was substantiated by the INADEQUATE spectrum. The absolute configuration of C-3′ was determined by application of the PGME (phenylglycine methyl ester) method[13] using di-(*R*)-PGME and di-(*S*)-PGME derivatives[14] of **1** to be *S*.

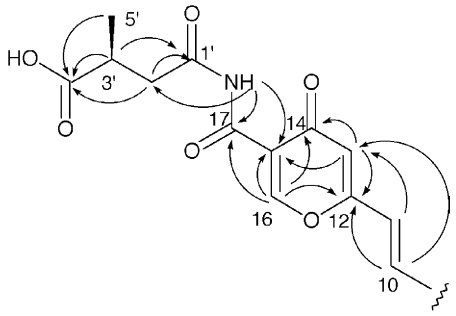


Figure 1. Key HMBC correlations for **1**.

Himeic acid B (**2**) had a molecular formula smaller than that of **1** by a $C_5H_6O_3$ unit. The ^1H and ^{13}C NMR spectra of **2** (Table 2) were almost superimposable on those of **1** except for the absence of unit B (C-1′–C-5′) and the presence of two prominent broad singlet protons at δ 7.88 and 9.19, which exhibited a weak cross-peak in the COSY spectrum. Interpretation of the 2D NMR data of **2** led to the structure of **2**.

Himeic acid C (**3**) had a molecular ion peak at *m*/*z* 435 $[M+H]^+$ in the FABMS, which was one mass unit less than that of **1** and matched a formula of $C_{22}H_{30}N_2O_7$. Although the ^1H and ^{13}C NMR spectra of **3** (Table 3) were very similar to those of **1**, differences in the chemical shift values were observed in C-10–C-17. Especially, the carbon signals at C-12 (δ 147.6) and C-16 (δ 143.7) were resonated in higher field than those of **1** (δ 162.6, C-12; δ 162.2, C-16), which were secured by analysis of 2D NMR data. These data suggested that the 4-pyrone ring in **1** was replaced by a 4-pyridone ring in **3**.

E1 catalyzes the formation of a ubiquitin–adenylate intermediate from ubiquitin and ATP, and subsequently the binding of ubiquitin to a cysteine residue in the E1 active site in a thiol ester linkage.[15–18] The effect of himeic acid A (**1**) on the formation of the E1–ubiquitin intermediate from a recombinant Flag-tagged human E1[19] and GST–ubiquitin in the presence of ATP was analyzed by Western blotting with anti-Flag antibody (Fig. 2).[20] Himeic acid A (**1**) inhibited the E1–ubiquitin

Table 2. NMR spectral data for **2** in $CDCl_3$–C_5D_5N (1:1)

	δ_H (mult., Hz)		δ_C	HMBC (#C)
1			176.3 C	
2	2.39 (2H)	t, 7.3	34.8 CH_2	1, 3
3	1.71 (2H)	Quintet, 7.3	25.3 CH_2	1, 2
4	1.37 (2H)	m	29.2[a] CH_2	
5	1.28 (2H)	m	29.34[a] CH_2	
6	1.28 (2H)	m	29.36[a] CH_2	
7	1.28 (2H)	m	29.39[a] CH_2	
8	1.41 (2H)	m	28.4 CH_2	
9	2.16 (2H)	q, 7.3	32.9 CH_2	8, 10, 11
10	6.65	dt, 15.9, 7.3	149.7 CH	8, 11, 12
11	6.05	d, 15.9	121.3 CH	9, 12, 13
12			162.7 C	
13	6.29	s	113.9 CH	11, 12, 15
14			178.5 C	
15			119.8 C	
16	8.77	s	161.3 CH	12, 14, 15, 17
17			164.4 C	
NH_2	7.88	br s		
	9.19	br s		

[a] May be interchangeable.

Table 3. NMR spectral data for **3** in $CDCl_3$–C_5D_5N (1:1)

	δ_H (mult., Hz)		δ_C	HMBC (#C)
1			176.1 C	
2	2.44 (2H)	t, 7.3	34.8 CH_2	1, 3
3	1.72 (2H)	Quintet, 7.3	25.5 CH_2	1, 2
4	1.35 (2H)	m	28.6[b] CH_2	
5	1.17[a] (2H)	m	29.1[b] CH_2	
6	1.22[a] (2H)	m	29.38[b] CH_2	
7	1.33[a] (2H)	m	29.44[b] CH_2	
8	1.30 (2H)	m	29.44[b] CH_2	
9	2.10 (2H)	dt, 7.0, 7.3	33.0 CH_2	10, 11
10	6.62	dt, 16.5, 7.3	139.9 CH	9, 12
11	6.24	d, 16.5	123.0 CH	9, 12, 13
12			147.6 C	
13	6.71	s	117.4 CH	11, 12, 14, 15
14			178.8 C	
15			116.9 C	
16	8.69	s	143.7 CH	12, 14, 17
17			164.2 C	
1′			174.0 C	
2′	3.13	dd, 17.2, 5.5	42.7 CH_2	1′, 4′, 5′
	3.69	dd, 17.2, 8.3		1′, 3′, 4′, 5′
3′	3.35	ddq, 8.3, 5.5, 6.3	35.9 CH	4′, 5′
5′	1.39 (3H)	d, 6.3	17.7 CH_3	2′, 3′, 4′
4′			178.2 C	
NH	13.70	s		17

[a] May be interchangeable.
[b] May be interchangeable.

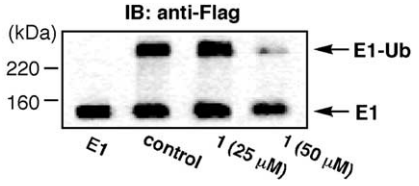


Figure 2. Inhibition of the E1–ubiquitin intermediate formation by **1**. Recombinant Flag-tagged human E1, GST–ubiquitin (Ub), and ATP were incubated in the presence or absence of 25 or 50 μM **1**, and the reaction mixture was then subjected to SDS-PAGE followed by immunoblotting (IB) with anti-Flag antibody and detection by the chemiluminescence method.

intermediate formation in a dose-dependent manner, and densitometric analysis revealed that the E1–ubiquitin intermediate formation was 65% inhibited by **1** at the concentration of 50 μM. On the other hand, himeic acids B (**2**) and C (**3**) were unable to inhibit the intermediate formation even at 100 μM. It is likely that a thiol group of the active site cysteine residue in E1 readily attacks an imide carbon C-1′ in **1**, resulting in inhibition of the thiol ester intermediate formation. However, **3** did not exhibit inhibitory activity in spite of the presence of the corresponding imide system in **3**. Details of the inhibitory mechanism of **1** are now under investigation.

It is well known that post-translational protein modifications, including protein phosphorylation, acetylation, methylation, and ubiquitination, regulate various cellular events. Among them, ubiquitination has been known as a signal for protein degradation by the 26S proteasome.[1–4] In addition to the role of ubiquitin as a tag for proteasomal degradation, accumulating evidence reveals that ubiquitin plays proteasome-independent roles in protein trafficking, endocytosis, DNA repair, and so on.[21] In consequence, ubiquitin is involved in a variety of proteasome-dependent and -independent cellular events, such as cell cycle progression, transcription, DNA repair, signal transduction, endocytosis, apoptosis, and the immune response.[1–4,21] Thus, ubiquitination has attracted widespread attention because of its wide regulatory roles. As bortezomib (PS-341), a proteasome inhibitor, has recently been approved by FDA for multiple myeloma treatment,[5] E1 inhibitors including himeic acid A (**1**) also could become lead compounds for treatment of diseases related to ubiquitination.

Two cognate compounds, designated as microsphaerones A and B, were isolated from a sponge-derived fungus, *Microsphaeropsis* sp.[22] Microsphaerones A and B have additional double bonds at C-8 in **1** and **2**, respectively, and no significant biological activity of them has been reported. In this study, we have reported the isolation, structure determination, and inhibitory activity against ubiquitin-activating enzyme of **1**. Although several proteasome inhibitors have been isolated from the natural resources, this is the second compound identified as the ubiquitin-activating enzyme inhibitor. The first compound was isolated from a mushroom strain, *Panus rudis* Fr. IFO8994.[23]

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan and the Ichiro Kanehara Foundation.

References and notes

1. Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425–479.
2. Weissman, A. M. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2*, 169–178.
3. Pickart, C. M. *Annu. Rev. Biochem.* **2001**, *70*, 503–533.
4. Glickman, M. H.; Ciechanover, A. *Physiol. Rev.* **2002**, *82*, 373–428.
5. Adams, J. *Drug Discovery Today* **2003**, *8*, 307–315.
6. Tsukamoto, S.; Tatsuno, M.; van Soest, R. W. M.; Yokosawa, H.; Ohta, T. *J. Nat. Prod.* **2003**, *66*, 1181–1185.
7. Tsukamoto, S.; Yamashita, K.; Tane, K.; Kizu, R.; Ohta, T.; Matsunaga, S.; Fusetani, N.; Kawahara, H.; Yokosawa, H. *Biol. Pharm. Bull.* **2004**, *27*, 699–701.
8. The fungus was identified on the basis of the morphological evaluation by NCIBM Japan Co., Ltd (Shizuoka, Japan). A voucher specimen is deposited at Kanazawa University with the code MF275.
9. The fungus was grown in a fermentation broth composed of 1:1 artificial seawater/deionized water with 2.0% malt extract and 0.5% peptone at 28 °C for 8 days.
10. Compound **1**: $[\alpha]_D^{26}$ −15 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 234.5 (4.5) and 254.0 nm (4.4); IR (film) ν_{max} 2975, 2818, 1747, 1727, 1694, 1647, 1622, 1572, 1502, 1409, 1161 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$–C_5D_5N 1:1) see

Table 1. FABMS (positive, glycerol matrix) *m/z* 322 $[M+H-115]^+$, 436 $[M+H]^+$, 458 $[M+Na]^+$; HRFABMS *m/z* 436.1977 (calcd for $C_{22}H_{30}NO_8$ 436.1971).

11. Compound **2**: UV (MeOH) λ_{max} ($\log\varepsilon$) 227.0 (4.3) and 266.0 nm (4.1); IR (film) ν_{max} 2918, 2846, 1697, 1668, 1558, 1541, 1409, 1251, 1188 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$–C_5D_5N 1:1) see Table 2. FABMS (positive, glycerol matrix) *m/z* 322 $[M+H]^+$; HRFABMS *m/z* 322.1651 (calcd for $C_{17}H_{24}NO_5$ 322.1654).
12. Compound **3**: $[\alpha]_D^{26}$ −9.8 (*c* 0.32, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 252.0 (4.4); IR (film) ν_{max} 2923, 2852, 1732, 1717, 1624, 1506, 1243, 1186 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$–C_5D_5N 1:1) see Table 3. FABMS (positive, glycerol matrix) *m/z* 435 $[M+H]^+$; HRFABMS *m/z* 435.2139 (calcd for $C_{22}H_{31}N_2O_7$ 435.2132).
13. Yabuuchi, T.; Kusumi, T. *J. Org. Chem.* **2000**, *65*, 397–404.
14. Di-(*S*)-PGME amide (**4**): ^1H NMR ($CDCl_3$): δ 1.21 (3H, d, J = 7.3 Hz, H_3-5′), 2.23 (1H, m, H-2), 2.24 (1H, m, H-2), 2.27 (2H, dt, J = 6.4, 7.3 Hz, H_2-9), 2.82 (1H, dd, J = 17.6, 4.9 Hz, H-2′), 2.97 (1H, m, H-3′), 3.32 (1H, dd, J = 17.6, 8.3 Hz, H-2′), 3.71 (3H, s, OMe), 3.73 (3H, s, OMe), 5.53 (1H, d, J = 6.9 Hz, CH of PGME moiety), 5.59 (1H, d, J = 6.9 Hz, CH of PGME moiety), 6.10 (1H, d, J = 16.0 Hz, H-11), 6.29 (1H, s, H-13), 6.38 (1H, d, J = 6.9 Hz, NH of PGME moiety), 6.77 (1H, dt, J = 16.0, 7.0 Hz, H-10), 6.95 (1H, d, J = 6.9 Hz, NH of PGME moiety), 8.73 (1H, s, H-16), 11.84 (1H, s, NH); FABMS (positive, glycerol matrix) *m/z* 762 $[M+H]^+$. Di-(*R*)-PGME amide (**5**): ^1H NMR ($CDCl_3$): δ 1.27 (3H, d, J = 6.9 Hz, H_3-5′), 2.23 (1H, m, H-2), 2.24 (1H, m, H-2), 2.27 (2H, dt, J = 6.4, 7.3 Hz, H_2-9), 2.79 (1H, dd, J = 17.6, 4.9 Hz, H-2′), 2.97 (1H, m, H-3′), 3.27 (1H, dd, J = 17.6, 8.8 Hz, H-2′), 3.730 (3H, s, OMe), 3.733 (3H, s, OMe), 5.55 (1H, d, J = 7.3 Hz, CH of PGME moiety), 5.59 (1H, d, J = 7.3 Hz, CH of PGME moiety), 6.09 (1H, d, J = 16.0 Hz, H-11), 6.28 (1H, s, H-13), 6.36 (1H, d, J = 7.3 Hz, NH of PGME moiety), 6.776 (1H, dt, J = 16.0, 7.0 Hz, H-10), 6.779 (1H, d, J = 7.3 Hz, NH of PGME moiety), 8.72 (1H, s, H-16), 11.77 (1H, s, NH); FABMS (positive, glycerol matrix) *m/z* 762 $[M+H]^+$.
15. Haas, A.; Warms, J.; Hershko, A.; Rose, I. *J. Biol. Chem.* **1982**, *257*, 2543–2548.
16. Haas, A.; Rose, I. *J. Biol. Chem.* **1982**, *257*, 10329–10337.
17. Walden, H.; Podgorski, M. S.; Schulman, B. A. *Nature* **2003**, *422*, 330–334.
18. Walden, H.; Podgorski, M. S.; Huang, D. T.; Miller, D. W.; Howard, R. J.; Minor, D. L., Jr.; Holton, J. M.; Schulman, B. A. *Mol. Cell.* **2003**, *12*, 1427–1437.
19. Saeki, Y.; Tayama, Y.; Toh-e, A.; Yokosawa, H. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 840–845.
20. E1 activity was measured on the basis of the formation of the E1–ubiquitin intermediate from E1 and ubiquitin in the presence of ATP. Flag-tagged E1[19] (0.1 μg) was previously incubated at 37 °C for 30 min in 25 μL of a reaction mixture containing 50 mM Tris–HCl, pH 7.6, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM ATP, and 0.25 units of inorganic pyrophosphatase (Sigma), and subsequently 0.5 μL GST–Ub (MBL) was added to the mixture and the resulting mixture was incubated at 37 °C for 60 min. The reaction was terminated by the addition of SDS-loading buffer and the reaction mixture was then subjected to SDS-PAGE in a slab gel containing 7% polyacrylamide under nonreducing conditions. After the proteins were blotted to the nitrocellurose membranes (BIO-RAD), blocking with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 and the subsequent immunoblotting were carried out. For immunochemical detection of Flag-tagged E1, a mouse monoclonal M2 antibody against Flag-tag (SIGMA) and peroxidase-conjugated anti-mouse IgG (Amersham) were used as the first and second antibodies, respectively. Detection was performed using an enhanced chemiluminescence system (Amersham), and bands were visualized with a BIO-RAD Fluor-S™ MultiImager and analyzed by a BIO-RAD Image Analysis System.
21. Hicke, L. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 195–201.
22. Wang, C.-Y.; Wang, B.-G.; Brauers, G.; Guan, H.-S.; Proksch, P.; Ebel, R. *J. Nat. Prod.* **2002**, *65*, 772–775.
23. Sekizawa, R.; Ikeno, S.; Nakamura, H.; Naganawa, H.; Matsui, S.; Iinuma, H.; Takeuchi, T. *J. Nat. Prod.* **2002**, *65*, 1491–1493.