Himeic Acids E–G, New 4-Pyridone Derivatives from a Culture of *Aspergillus* sp.

Toshiyuki Kuwana, Mitsue Miyazaki, Hikaru Kato, and Sachiko Tsukamoto*

Graduate School of Pharmaceutical Sciences, Kumamoto University; Kumamoto 862–0973, Japan.
Received September 12, 2012; accepted October 24, 2012

Three new himeic acids E–G were isolated from a marine-derived fungus, *Aspergillus* sp., and their structures were determined by spectroscopic analysis. Although himeic acid A inhibited the activity of ubiquitin-activating enzyme (E1), the three new derivatives did not.

Key words 4-pyridone derivative; ubiquitin-activating enzyme (E1); fungus

Protein degradation is regulated by the ubiquitin-proteasome system and is essential for various cellular events including cell-cycle control, transcription, and development.[1,2] The ubiquitin-proteasome system consists of the ubiquitin system, which is composed of three enzymes [ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3)] and catalyzes ubiquitination of client proteins, and the 26S proteasome, which degrades polyubiquitinated client proteins.[1–6] In our search for anticancer agents targeting the ubiquitin-proteasome system, we isolated himeic acid A (**1**)[7] (Fig. 1) from the culture of a marine-derived *Aspergillus* sp. and hyrtioreticulin A[8] from the marine sponge *Hyrtios reticulatus* as E1 inhibitors. E1 activity is essential for the ubiquitin-proteasome system and, therefore, inhibitors against E1 would be another powerful agent for the treatment of cancer. We isolated **1** together with himeic acids B (**2**) and C (**3**) from the *Aspergillus* culture. In spite of their structural similarities, their inhibitory effects differed.[7] While **1** caused 65% inhibition at 50 μM, **2** and **3** had no effect even at 100 μM. Since **1** did not inhibit E1-like enzymes for other ubiquitin-like modifiers, namely, SUMO-1 and ISG15, it seems to be a specific inhibitor of the ubiquitin E1 enzyme. In the continuing search for more potent E1 inhibitors, we tried to find minor congeners of himeic acids in the culture. Here, we report the isolation and structural elucidation of himeic acids E–G (**4**–**6**).

The fungus was cultured, and then the mycelium and broth were separated by filtration. The mycelium was extracted with EtOAc and the concentrated extract was partitioned between *n*-hexane and 90% MeOH–H_2O. The culture broth was extracted with *n*-BuOH and the extract was partitioned between *n*-hexane and 90% MeOH–H_2O. The two aqueous MeOH fractions were combined and subjected to octadecyl silica (ODS) column chromatography with MeOH–H_2O (20, 40, 60, 80, 100%). The fractions eluted with 60 and 80% MeOH–H_2O were also combined, and repeatedly purified by ODS HPLC with MeCN–H_2O–trifluoroacetic acid (TFA) to afford **4**–**6** together with **1**–**3** and microsphaerone C (**7**).[9]

High resolution-electrospray ionization (HR-ESI)-MS showed the molecular formula of **4** to be $C_{22}H_{30}N_2O_7$, which is identical to that of **3**. The ^{1}H- and ^{13}C-NMR spectra in DMSO-d_6 (Table 1) were almost superimposable on those of **3** except for the coupling pattern of olefinic signals. H-10 and H-11 were observed at δ 6.07 (dt, J=11.9, 7.3 Hz) and 6.22 (d, J=11.9 Hz), respectively. The magnitude of the coupling constant showed that **4** has a *cis* double bond, in contrast to *trans* in **3**. The specific rotation of **4** was −12°, the sign of which was the same as for **1** and **3** (−15° and −9.8°, respectively). Accordingly, the absolute configuration of C-3′ of **4** was indicated to be *S* based on biogenetic considerations. Therefore, **4** was shown to be a geometrical isomer of **3**.

The molecular formula of **5** was established by HR-ESI-MS as $C_{17}H_{24}N_2O_4$, $C_5H_6O_3$ less than that of **4**. The ^{1}H- and ^{13}C-NMR spectra of **5** (Table 2) readily showed the presence of two mutually coupled signals at δ 7.44 (d, J=4.3 Hz) and 9.53 (d, J=4.3 Hz) instead of the absence of a unit X, C-1′–C-5′ (see Fig. 1) and a singlet signal at δ 13.06 (17-NH) compared to **4**. The remaining unit, C-1–C-17, was identical to the corresponding unit of **4**, which was unambiguously established by 2 dimensional (2D)-NMR spectra. These data suggested that the unit X in **4** was replaced by a hydrogen atom in **5**.

The ^{1}H- and ^{13}C-NMR spectra of **6** (Table 2) were similar to **5** except for the presence of a methyl group (δ_H 3.56 (3H, s) and δ_C 51.2 (qC)). Heteronuclear multiple bond connectivity (HMBC) cross peaks from δ 3.56, 2.27 (H_2-2), and 1.50 (H_2-3) to δ 173.4 (C-1) showed that the additional methyl group was accommodated on a methyl ester at C-1. The coupling constant of olefinic hydrogens at δ 6.25 and 6.63 was 16.1 Hz, which suggested the *trans*-configuration. The molecular formula $C_{18}H_{26}N_2O_4$ indicated by HR-ESI-MS supported the structure of **6** analyzed using the NMR data.

The effects of himeic acids E–G (**4**–**6**) and microsphaerone C (**7**) on the formation of the E1-ubiquitin intermediate were tested by Western blotting.[7,8] They were unable to inhibit the intermediate's formation even at 200 μM. These results clearly showed that both a 4-pyrone ring and unit X (C-1′–C-5′) were essential to inhibit formation of the E1-ubiquitin intermediate and confirmed the previous result that himeic acid A (**1**), but not B (**2**) or C (**3**), inhibited E1 activity.[7]

Despite many attempts to develop E1 inhibitors, only four natural inhibitors, panapophenanthorine,[10] himeic acid A,[7] largazole,[11] and hyrtioreticulin A,[8] and two synthetic inhibitors, PYR-41[12] and NSC624206,[13] have been discovered so far. Among them, PYR-41 is cell-permeable and blocks protein degradation and cytokine-induced activation of nuclear factor-kappa B (NF-κB), which leads to the activation of p53 in cells and death of cells transformed with wild-type p53. Nowadays, inhibitors targeting the ubiquitin system including E1, E2, and E3 enzymes, the delivery system, and deubiquitinating

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: sachiko@kumamoto-u.ac.jp

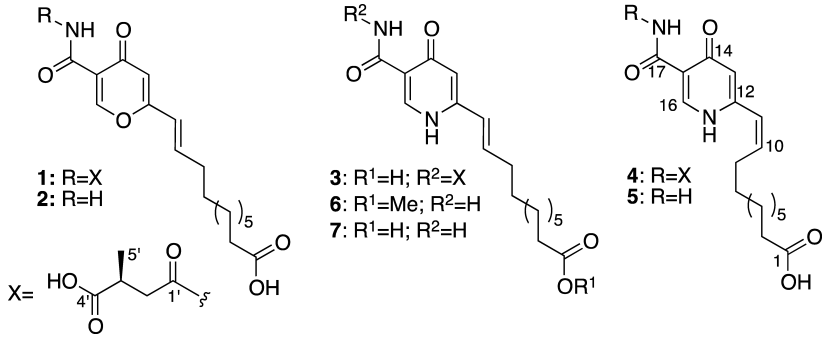

Fig. 1. Structures of **1**–**7**

Table 1. ^{1}H- and ^{13}C-NMR Data for **4** in DMSO-d_6

	δ_H	δ_C
1		174.5
2	2.16 t 7.3	33.7
3	1.45 m	24.5
4	1.22 m	28.5[a)]
5	1.22 m	28.6[a)]
6	1.22 m	28.7[a)]
7	1.22 m	28.7[a)]
8	1.41 m	28.7[a)]
9	2.29 q 7.3	28.7[a)]
10	6.07 dt 11.9, 7.3	141.5
11	6.22 d 11.9	120.9
12		146.8
13	6.40 s	118.4
14		177.2
15		115.1
16	8.47 s	143.6
17		163.1
1′		173.2
2′	2.81 dd 17.5, 5.0	41.9
	3.12 dd 17.5, 8.4	
3′	2.78 ddq 8.4, 5.0, 6.9	34.5
4′		176.7
5′	1.13 s 6.9	17.1
12-NH	12.13 brs	
17-NH	13.06 s	

a) May be interchangeable.

enzymes are also candidates for anticancer drugs, and several compounds are now undergoing preclinical and clinical trials.[14–16)] Although a NEDD8 E1 inhibitor, MLN4924,[17,18)] is now under phase I/II trials, no ubiquitin-E1 inhibitor has been evaluated yet. The discovery of ubiquitin-E1 inhibitors is urgently needed to develop more efficient anticancer drugs and to investigate the complex ubiquitin-proteasome system.

Experimental

Optical rotation was determined with a JASCO P-1000 polarimeter in MeOH. UV spectra were measured on a JASCO V-550 spectrophotometer in MeOH. IR spectra were measured on a JEOL JIR-6500W spectrophotometer. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer in DMSO-d_6. Chemical shifts in DMSO-d_6 were referenced to the residual solvent peaks, δ_H 2.49 and δ_C 39.5. Mass spectra were measured on a Bruker esquire3000plus-K1 or Bruker Bio-TOF mass spectrometer.

Extraction and Isolation The strain of *Aspergillus* sp. (MF275),[7)] which was isolated from mussel, was cultured in a medium (40 mL×14) composed of 2.0% malt extract and 0.5% peptone in 50% seawater at 25°C with shaking for two weeks. The mycelium and culture broth were separated by filtration. The mycelium was extracted with EtOAc and the concentrated extract (337.9 mg) was partitioned between *n*-hexane (186.5 mg) and 90% MeOH–H_2O (125.5 mg). The culture broth was extracted with *n*-BuOH and the extract (930.8 mg) was partitioned between *n*-hexane (262.0 mg) and 90% MeOH–H_2O (645.3 mg). The two aqueous MeOH fractions were combined and subjected to ODS column chromatography with MeOH–H_2O (20, 40, 60, 80, 100%). The fractions (102.2 mg) eluted with 60 and 80% MeOH–H_2O were also combined and repeatedly purified by ODS HPLC with MeCN–H_2O–TFA (27.5–35% MeCN–H_2O containing 0.05% TFA) to afford himeic acids E (**4**, 0.98 mg), F (**5**, 1.28 mg), and G (**6**, 0.98 mg) together with himeic acids A (**1**, 17.4 mg), B (**2**, 0.80 mg), and C (**3**, 55.6 mg) and microsphaerone C (**7**, 3.0 mg).[9)]

4: ^{1}H- and ^{13}C-NMR (DMSO-d_6) see Table 1. HMBC correlations: H_2-2/C-1, C-3, C-4; H_2-3/C-1; H_2-8/C-10; H_2-9/C-10, C-11; H-10/C-8, C-9, C-12; H-11/C-9, C-10, C-13; H-13/C-11, C-12, C-14, C-15; H-16/C-12, C-14, C-15, C-17; H-2′ (δ 2.81)/C-1′, C-3′, C-5′; H-2′ (δ 3.12)/C-1′, C-4′; H-3′/C-4′; H-5′/C-2′, C-3′, C-4′; 17-NH/C-2′. IR (film) cm^{-1}: 2927, 2852, 1734, 1716, 1699, 1684, 1653, 1558, 1541, 1506, 1458, 1205, 1182, 1134. UV λ_{max} (MeOH) nm (logε): 300 (sh, 3.1), 249 (3.9). HR-ESI-MS *m/z*: 433.1965 (M−H)$^-$ (Calcd for $C_{22}H_{29}N_2O_7$: 433.1975). ESI-MS *m/z*: 433 (M−H)$^-$. $[\alpha]_D^{20}$ −12° (*c*=0.89, MeOH).

5: ^{1}H- and ^{13}C-NMR (DMSO-d_6) see Table 2. HMBC correlations: H_2-2/C-3; H_2-9/C-10, C-11; H-10/C-9, C-11, C-12; H-11/C-9, C-10, C-13; H-13/C-11, C-12, C-15; H-16/C-12, C-14. IR (film) cm^{-1}: 2927, 2854, 1734, 1716, 1699, 1684, 1653, 1558, 1541, 1506, 1458, 1205, 1182, 1136. UV λ_{max} (MeOH) nm (logε): 230 (3.2). HR-ESI-MS *m/z*: 319.1662 (M−H)$^-$ (Calcd for $C_{17}H_{23}N_2O_4$: 319.1658). ESI-MS *m/z*: 319 (M−H)$^-$.

6: ^{1}H- and ^{13}C-NMR (DMSO-d_6) see Table 2. HMBC correlations: H_2-2/C-1, C-3; H_2-3/C-1; H_2-8/C-10; H_2-9/C-8, C-10, C-11; H-10/C-8, C-9, C-12; H-11/C-9, C-12, C-13; H-13/C-11, C-12, C-15; H-16/C-17; 1-OMe/C-1; 12-NH/C-13, C-15; 17-NH_2/C-15. IR (film) cm^{-1}: 2924, 2854, 1734, 1716, 1699, 1684, 1670, 1653, 1635, 1558, 1541, 1522, 1508, 1207, 1182, 1134. UV λ_{max} (MeOH) nm (logε): 297 (sh, 3.1), 246 (3.9). HR-ESI-MS *m/z*: 333.1777 (M−H)$^-$ (Calcd for $C_{18}H_{25}N_2O_4$: 333.1814). ESI-MS *m/z*: 333 (M−H)$^-$.

Measurement of E1 Activity This assay was performed as described previously.[7,8)]

Table 2. ^{1}H- and ^{13}C-NMR Data for **5** and **6** in DMSO-d_6

	5		**6**	
	δ_H	δ_C	δ_H	δ_C
1		174.6		173.4
2	2.16 t 7.3	33.6	2.27 t 7.4	33.3
3	1.45 m	24.5	1.50 m	24.4
4	1.22 m	28.5[a)]	1.25 m	28.45[a)]
5	1.22 m	28.6[a)]	1.25 m	28.48[a)]
6	1.22 m	28.7[a)]	1.25 m	28.6[a)]
7	1.22 m	28.7[a)]	1.25 m	28.7[a)]
8	1.40 m	28.7[a)]	1.42 m	28.1
9	2.26 q 7.3	28.7[a)]	2.20 q 7.3	32.3
10	6.03 dt 11.8, 7.3	140.7	6.63 dt 16.1, 7.3	138.5
11	6.18 d 11.8	121.0	6.25 d 16.1	122.6
12		145.3		145.7
13	6.28 s	118.2	6.39 s	116.0
14		177.3		177.7
15		117.3		117.7
16	8.32 s	141.5	8.26 d 6.2	141.5
17		165.4		165.4
1-OMe			3.56 s	51.2
12-NH	12.08 br s		12.06 d 6.2	
17-NH_2	7.44 d 4.3		7.43 d 4.5	
	9.53 d 4.3		9.52 d 4.5	

a) May be interchangeable in each column.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research (No. 22310138) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and also by Grants from the Naito Foundation, the Astellas Foundation for Research on Metabolic Disorders, and the Uehara Memorial Foundation.

References

1) Hershko A., Ciechanover A., *Annu. Rev. Biochem.*, **67**, 425–479 (1998).
2) Glickman M. H., Ciechanover A., *Physiol. Rev.*, **82**, 373–428 (2002).
3) Voges D., Zwickl P., Baumeister W., *Annu. Rev. Biochem.*, **68**, 1015–1068 (1999).
4) Pickart C. M., *Annu. Rev. Biochem.*, **70**, 503–533 (2001).
5) Finley D., *Annu. Rev. Biochem.*, **78**, 477–513 (2009).
6) Tanaka K., *Proc. Jpn. Acad. B: Phys. Biol. Sci.*, **85**, 12–36 (2009).
7) Tsukamoto S., Hirota H., Imachi M., Fujimuro M., Onuki H., Ohta T., Yokosawa H., *Bioorg. Med. Chem. Lett.*, **15**, 191–194 (2005).
8) Yamanokuchi R., Imada K., Miyazaki M., Kato H., Watanabe T., Fujimuro M., Saeki Y., Yoshinaga S., Terasawa H., Iwasaki N., Rotinsulu H., Losung F., Mangindaan R. E. P., Namikoshi M., de Voogd N. J., Yokosawa H., Tsukamoto S., *Bioorg. Med. Chem.*, **20**, 4437–4442 (2012).
9) Sowemimo A. A., Edrada-Ebel R., Ebel R., Proksch P., Omobuwajo O. R., Adesanya S. A., *Nat. Prod. Commun.*, **3**, 1217–1222 (2008).
10) Sekizawa R., Ikeno S., Nakamura H., Naganawa H., Matsui S., Iinuma H., Takeuchi T., *J. Nat. Prod.*, **65**, 1491–1493 (2002).
11) Ungermannova D., Parker S. J., Nasveschuk C. G., Wang W., Quade B., Zhang G., Kuchta R. D., Phillips A. J., Liu X., *PLoS ONE*, **7**, e29208 (2012).
12) Yang Y., Kitagaki J., Dai R. M., Tsai Y. C., Lorick K. L., Ludwig R. L., Pierre S. A., Jensen J. P., Davydov I. V., Oberoi P., Li C. C., Kenten J. H., Beutler J. A., Vousden K. H., Weissman A. M., *Cancer Res.*, **67**, 9472–9481 (2007).
13) Ungermannova D., Parker S. J., Nasveschuk C. G., Chapnick D. A., Phillips A. J., Kuchta R. D., Liu X., *J. Biomol. Screen.*, **17**, 421–434 (2012).
14) Bedford L., Lowe J., Dick L. R., Mayer R. J., Brownell J. E., *Nat. Rev. Drug Discov.*, **10**, 29–46 (2011).
15) Cohen P., Tcherpakov M., *Cell*, **143**, 686–693 (2010).
16) Ande S. R., Chen J., Maddika S., *Eur. J. Pharmacol.*, **625**, 199–205 (2009).
17) Soucy T. A., Smith P. G., Milhollen M. A., Berger A. J., Gavin J. M., Adhikari S., Brownell J. E., Burke K. E., Cardin D. P., Critchley S., Cullis C. A., Doucette A., Garnsey J. J., Gaulin J. L., Gershman R. E., Lublinsky A. R., McDonald A., Mizutani H., Narayanan U., Olhava E. J., Peluso S., Rezaei M., Sintchak M. D., Talreja T., Thomas M. P., Traore T., Vyskocil S., Weatherhead G. S., Yu J., Zhang J., Dick L. R., Claiborne C. F., Rolfe M., Bolen J. B., Langston S. P., *Nature*, **458**, 732–736 (2009).
18) Brownell J. E., Sintchak M. D., Gavin J. M., Liao H., Bruzzese F. J., Bump N. J., Soucy T. A., Milhollen M. A., Yang X., Burkhardt A. L., Ma J., Loke H. K., Lingaraj T., Wu D., Hamman K. B., Spelman J. J., Cullis C. A., Langston S. P., Vyskocil S., Sells T. B., Mallender W. D., Visiers I., Li P., Claiborne C. F., Rolfe M., Bolen J. B., Dick L. R., *Mol. Cell*, **37**, 102–111 (2010).