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Leucettamol A: A new inhibitor of Ubc13-Uev1A interaction isolated from a marine sponge, *Leucetta* aff. *microrhaphis*

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ABSTRACT

A compound that inhibits the formation of a complex composed of the ubiquitin E2 enzyme Ubc13 and Uev1A was isolated from the marine sponge *Leucetta* aff. *microrhaphis*. The compound was identified as leucettamol A (**1**) by spectroscopic analysis. Its inhibition of Ubc13-Uev1A interaction was tested by the ELISA method, revealing an IC_{50} value of 50 μg/mL. The compound is the first inhibitor of Ubc13-Uev1A interaction, that is, that of the E2 activity of Ubc13. Such inhibitors are presumed to be leads for anti-cancer agents that upregulate activity of the tumor suppressor p53 protein. Interestingly, hydrogenation of **1** increased its inhibitory activity with an IC_{50} value of 4 μg/mL, while its tetraacetate derivative was inactive, indicating that the hydroxy and/or amino groups of **1** are required for the inhibition.

The successful use of a proteasome inhibitor, for example, Velcade®,[1] in anti-cancer therapy led to the development of new drugs targeting the ubiquitin–proteasome system, an intracellular ubiquitin-dependent proteolytic pathway that plays important roles in various cellular events.[2–4] In the ubiquitin-proteasome system, ubiquitin attaches to a target protein prior to degradation by the sequential action of three enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin–protein ligase (E3). The polyubiquitin chain linked to the target protein is recognized by the 26S proteasome, and the protein portion is degraded by the proteolytic active sites in the 26S proteasome. In addition to the search for proteasome inhibitors, the development of drugs inhibiting factors upstream of the 26S proteasome as anti-cancer agents has attracted much attention. Notably, the search for E3 inhibitors has been extensively carried out:[5] E3s are a large family of enzymes that recognize huge numbers of target proteins and definitively determine which proteins are ubiquitinated for degradation. A specific inhibitor against an E3 recognizing a key protein would be a good lead for treatment of a disease connected with the turnover of the key protein. For example, many kinds of inhibitors against MDM2/HMD2, an E3 for the tumor suppressor p53 protein, have been isolated from chemical libraries[6] and natural resources,[7,8] and also synthesized[9,10] as anti-cancer agents.

Ubiquitination performs proteolytic and non-proteolytic functions:[11,12] The lysine48 (K48)-linked polyubiquitin chain is related to proteasome-dependent protein degradation, while the K63-linked chain plays non-proteolytic roles in signal transduction and DNA repair. The formation of the latter chain is catalyzed by a heterodimer formed by the ubiquitin E2 enzyme Ubc13 and either Uev1A or MMS2,[13] and a functional difference between the two Ubc13 complexes was suggested.[14] Recently, Laine et al. reported that expression of Ubc13 induced the nuclear export of p53, resulting in a decrease in its transcriptional activity and that the knockdown of Ubc13 led to an increase in p53 activity.[15] This report led us to speculate that an inhibitor of Ubc13, that is, one preventing the formation of the Ubc13-Uev1A complex, would be a lead for an anti-cancer agent. In this study, we carried out a search for inhibitors of Ubc13-Uev1A interaction in natural resources and found leucettamol A (**1**) (Fig. 1) in the marine sponge *Leucetta* aff. *microrhaphis*.

Specimens of *Leucetta* aff. *microrhaphis* (Haeckel, 1872)[16] (80 g, wet weight) were collected in North Sulawesi, Indonesia, in September 2006. The sponge was extracted with EtOH immediately

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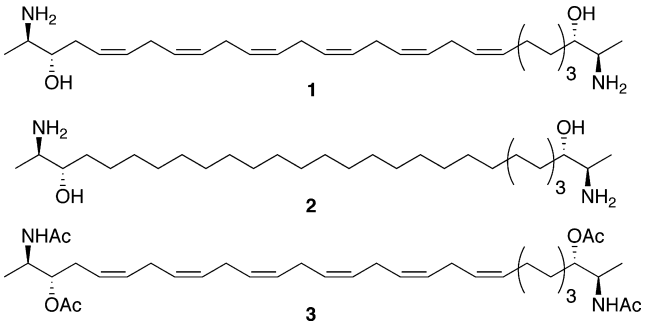

Figure 1. Structures of leucettamol A (**1**) and its derivatives (**2** and **3**).

after collection. The extract was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The *n*-BuOH fraction (0.61 g) showed inhibitory activity against Ubc13-Uev1A interaction and was subjected to ODS column chromatography with 40% CH_3CN–H_2O to afford leucettamol A (**1**, 202 mg, 0.25%), which was originally isolated from *Leucetta microrhaphis* collected in Bermuda as an antimicrobial compound[17] and was identified on the basis of spectroscopic data.[18] Although compound **1** had been reported as a racemic one in the original paper,[17] Molinski et al. recently found that this compound is a chiral one with the configuration of 2*R*,3*S*,28*S*,29*R*, revealed by the deconvoluted exciton coupled circular dichroism (ECCD) spectrum.[19]

The inhibition of Ubc13-Uev1A interaction was tested by ELISA according to standard procedures using purified recombinant Ubc13 and FLAG-Uev1A proteins[20] and a primary anti-FLAG antibody (SIGMA, F3165).[21] The interaction was inhibited by **1** with an IC_{50} value of 50 μg/mL. To preliminarily analyze the structure–activity relationship, two derivatives of **1**, a hydrogenated derivative **2**[22] and a tetraacetate **3**[23] (Fig. 1), were prepared and their inhibitory activities were measured. Compound **2** was more potent than **1** with an IC_{50} value of 4 μg/mL, and **3** was inactive. These results suggest that the hydroxy and/or amino groups of **1** are required for the inhibition. Ubc13 is capable of forming a heterodimer with Uev1A or MMS2, and Uev1A and MMS2 share >90% amino acid sequence identity to each other in their core domains.[14] In our preliminary study, we found that compound **1** was unable to inhibit the formation of the Ubc13-MMS2 complex, suggesting the strict inhibitory specificity of **1**. To our knowledge, this is the first report on a compound inhibiting the formation of the Ubc13-Uev1A complex. This compound could function as an inhibitor of the ubiquitin E2 activity of Ubc13 because Ubc13 forms an obligatory complex with Uev1A to exhibit its E2 activity. The crystal structure of the Ubc13-Uev1A complex bound to the U box domain of the ubiquitin E3 ligase CHIP has been determined.[24] It is worth determining whether compound **1** or its derivative would bind to the constituent(s) of the complex, Ubc13 and/or Uev1A, to inhibit the complex formation.

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16. The marine sponge was collected by scuba diving at a depth of 10 m in North Sulawesi, Indonesia in September 2006 and soaked in EtOH immediately. The sponge was identified as *Leucetta* aff. *microrhaphis* (Haeckel, 1872). A voucher specimen (ZMAPOR20126) was deposited at the Institute for Systematics and Ecology, University of Amsterdam, The Netherlands.
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18. **1**: $[\alpha]_D^{24}$ −3.8° (*c* 4.4, MeOH). ^{1}H NMR (CD_3OD) δ 1.21 (3H, d, *J* = 6.5 Hz, CH_3), 1.24 (3H, d, *J* = 6.5 Hz, CH_3), 1.36 (6H, m), 1.44 (2H, m), 1.51 (2H, m), 2.08 (2H, m), 2.28 (2H, m), 2.81 (2H, m), 2.85 (8H, m), 3.27 (1H, m), 3.29 (1H, m), 3.70 (1H, m), 3.79 (1H, m), 5.32 (1H, m), 5.37 (9H, m), 5.45 (1H, m), 5.50 (1H, m). ^{13}C NMR (CD_3OD) δ 11.9 (CH_3), 12.1 (CH_3), 26.5 (3C, CH_2), 26.7 (CH_2), 26.8 (CH_2), 28.1 (CH_2), 30.2 (CH_2), 30.4 (CH_2), 30.6 (CH_2), 32.3 (CH_2), 33.9 (CH_2), 52.0 (CH), 52.6 (CH), 71.4 (CH), 71.5 (CH), 125.9 (CH), 128.7 (CH), 128.79 (CH), 128.85 (CH), 128.97 (CH), 129.01 (CH), 129.1 (2C, CH), 129.3 (CH), 129.4 (CH), 131.1 (CH), 131.6 (CH). FABMS (positive) *m*/*z* 473 $[M+H]^+$.
19. Dalisay, D. S.; Tsukamoto, S.; Molinski, T. F., submitted for publication.
20. *Escherichia coli* BL21 cells transformed with pGEX6P1-Ubc13 or pGEX6P1-FLAG-Uev1A were precultured overnight at 37 °C in LB medium supplemented with 50 μg/ml ampicillin, transferred to a 20-fold volume of the same medium, and cultured at 37 °C for 3 h. Isopropyl 1-thio-β-D-galactoside was then added at a final concentration of 0.1 mM, and the cells were further cultured at 30 °C for 6 h. Two GST-fused proteins were purified by using glutathione-immobilized agarose beads and the GST tag of GST-Ubc13 or GST-FLAG-Uev1A was removed by cleavage with PreScission protease (GE Healthcare).
21. The inhibition of Ubc13-Uev1A interaction was tested by ELISA with 96-well plates (F96 maxisorp immuno plate) (Nunc). Human Ubc13 diluted in phosphate-buffered saline (PBS) was coated on to 96-well plates and incubated at 4 °C overnight. The wells were extensively washed with 0.05% Tween 20/PBS (PBST) and incubated with 5% bovine serum albumin (BSA) (Sigma)/PBS at 37 °C for 1.5 h. After washing with PBST, the wells were incubated for 1.5 h with a mixture of FLAG-Uev1A and a test sample diluted in PBS that had been previously incubated at 37 °C for 15 min. The wells were thoroughly washed with PBST and were then incubated with anti-FLAG M2 monoclonal antibody (Sigma) in 5% BSA/PBST for 1.5 h, followed by 1.5 h-incubation with the second antibody (mouse IgG-HRP) (Amasham) in 5% BSA/PBST. After washing with PBST and then citrate–phosphate buffer (pH 5.0), a mixture of *o*-phenylene diamine and 0.007% H_2O_2 in the citrate-phosphate buffer was added to the wells and the wells were incubated at 37 °C for 30 min. Finally, 2 M H_2SO_4 was added to the wells and the optical density at 490 nm was measured on a microplate reader. The IC_{50} value, the concentration required for 50% inhibition of Ubc13-Uev1A interaction, was calculated from the data of duplicate experiments.
22. Compound **1** (3.0 mg) was treated with palladium–charcol in MeOH (5 mL) under a hydrogen atmosphere at room temperature for 24 h. After filtration of the catalyst, the solvent was evaporated. The residue was subjected to chromatography on a silica gel column with 10% MeOH–$CHCl_3$ to afford **2** (2.6 mg). **2**: ^{1}H NMR (CD_3OD) δ 1.20 (6H, d, *J* = 7.0 Hz, 2× CH_3), 1.27–1.33 (44H, m), 1.43 (4H, m), 3.25 (2H, m), 3.68 (2H, m). FABMS (positive) *m*/*z* 485 $[M+H]^+$.
23. A solution of **1** (3.0 mg) in pyridine (1.0 mL) was added to acetic anhydride (0.8 mL), and the mixture was kept at room temperature for 24 h. The solvent was evaporated, and the residue was purified by silica gel chromatography with 5% MeOH–$CHCl_3$ to afford **3** (2.0 mg). **3**: ^{1}H NMR ($CDCl_3$) δ 1.07 (3H, d, *J* = 7.0 Hz, CH_3), 1.10 (3H, d, *J* = 7.0 Hz, CH_3), 1.27–1.33 (8H, m), 1.48 (1H, m), 1.54 (1H, m), 1.92 (6H, s, 2× CH_3CO), 2.02 (4H, m), 2.05 (3H, s, CH_3CO), 2.06 (3H, s, CH_3CO), 2.27 (1H, m), 2.40 (1H, m), 2.80 (8H, m), 4.13 (1H, m), 4.17 (1H, m), 4.81 (1H, m), 4.86 (1H, m), 5.34 (11H, m), 5.46 (1H, m), 5.86 (2H, br s, 2× NH). FABMS (positive) *m*/*z* 641 $[M+H]^+$.
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