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Enantiodifferentiation of ketoprofen by Japanese firefly luciferase from *Luciola lateralis*

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ABSTRACT

Recently, we found that firefly luciferase exhibited (*R*)-enantioselective thioesterification activity toward 2-arylpropanoic acids. In the case of Japanese firefly luciferase from *Luciola lateralis* (LUC-H), the *E*-value for ketoprofen was approximately 20. In this study, we used a spectrophotometric method to measure the catalytic activity of LUC-H. Using this method allowed us to judge the reaction efficiency easily. Our results confirmed that LUC-H exhibits enantioselective thioesterification activity toward a series of 2-arylpropanoic acids. The highest activity was observed with ketoprofen. We also observed high enzymatic activity of LUC-H toward long-chain fatty acids. These results were reasonable because LUC-H is homologous with long-chain acyl-CoA synthetase. To obtain further information about the enantiodifferentiation mechanism of the LUC-H catalyzed thioesterification of ketoprofen, we determined the kinetic parameters of the reaction relative to each of its three substrates: ketoprofen, ATP, and coenzyme A (CoASH). We found that whereas the affinities of each compound are not affected by the chirality of ketoprofen, enantiodifferentiation is achieved by a chirality-dependent difference in the k_{cat} parameter.

1. Introduction

Firefly luciferase catalyzes the oxidation of D-luciferin with molecular oxygen in the presence of ATP and Mg^{2+}, producing light [1–3]. This bioluminescence reaction occurs in two steps: adenylation of D-luciferin followed by oxidation. It has been shown that firefly luciferase can also catalyze thioesterification. In the presence of ATP, Mg^{2+}, and coenzyme A (CoASH), this enzyme converts the non-luminescent substrates L-luciferin and dehydroluciferin into the corresponding thioesters [4–6]. This catalytic activity allows firefly luciferase to recover from binding a non-luminescent substrate, resulting in an overall enhancement of the rate of the bioluminescence reaction. In addition, Oba et al. reported that firefly luciferases from *Luciola cruciata* and *Photinus pyralis* exhibit acyl-CoA synthetase-like activity toward long-chain fatty acids such as arachidonic acid [7]. These reports inspired us to investigate the application of firefly luciferase to synthetic substrates. We were intrigued by the resemblance between firefly luciferases and long-chain acyl-CoA synthetase (LACS). LACS is involved in a deracemization reaction and can catalyze the enantioselective thioesterification of 2-arylpropanoic acid [8,9]. Based on the sequence homology and the similarity of the reaction mechanisms between firefly luciferase and LACS [10], we predicted that the thioesterification activity of firefly luciferase would display enantioselectivity. We have demonstrated that firefly luciferases from *Luciola lateralis*, *Luciola cruciata*, and *Photinus pyralis* catalyze the thioesterification of a series of 2-arylpropanoic acids in an enantioselective manner, and the (*R*)-form is transformed more efficiently than the (*S*)-form (Fig. 1) [11]. Utilization of firefly luciferase would, therefore, be a viable new option for the preparation of optically active 2-arylpropanoic acids. In the case of Japanese firefly luciferase from *L. lateralis* (LUC-H), the *E*-value for ketoprofen was approximately 20. The thioesterification reaction occurs in two steps via the formation of an acyl-AMP intermediate, and LUC-H can distinguish the absolute configuration of ketoprofen in either step. The detailed enantiodifferentiation mechanism, however, remained unknown.

In this study, we used a spectrophotometric method to analyze the substrate specificity of recombinant LUC-H; this technique allowed us to judge the reaction efficiency easily. We also determined the kinetic parameters of the thioesterification reaction relative to each of its three substrates: ketoprofen, ATP, and CoASH. We compared the kinetic parameters obtained using each enantiomer of ketoprofen separately. We also conducted inhibition experiments using an acyl-AMP intermediate analogue, 5′-*O*-(*N*-ketoprofenylsulfamoyl)adenosine. Based on these results, we have

Abbreviations: CoASH, coenzyme A; LACS, long-chain acyl-CoA synthetase; LUC-H, Japanese firefly luciferase from *Luciola lateralis*; PPB, potassium phosphate buffer.

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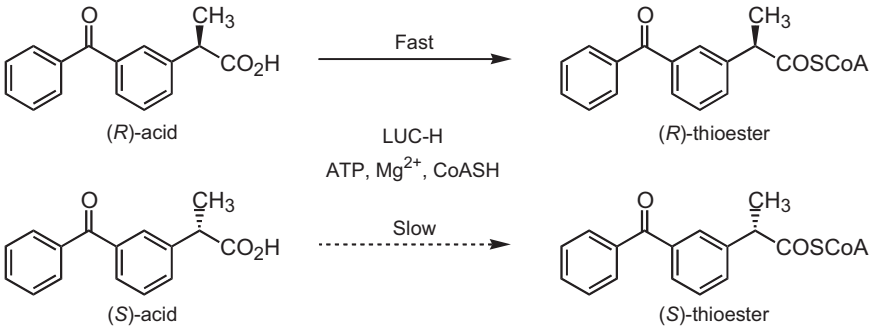

Fig. 1. LUC-H catalyzed enantioselective thioesterification of ketoprofen.

proposed a rationale for enantiodifferentiation in LUC-H-catalyzed thioester formation.

2. Materials and methods

2.1. General materials

The pHLfLK plasmid encoding the LUC-H gene was a kind gift from Kikkoman Corporation Ltd. [12]. (*R*)-Ketoprofen was prepared by enantioselective decarboxylation of 2-methyl-2-(3-benzoylphenyl)malonic acid using arylmalonate decarboxylases from *Mesorhizobium* sp. BNC1 [13]. Other chemicals were purchased from standard vendors and used without further purification unless otherwise noted. Protein concentrations were determined by a Bradford assay with bovine serum albumin as a standard [14].

2.2. Synthesis of the ketoprofenyl-AMP intermediate analogue, 5′-O-(N-ketoprofenylsulfamoyl)adenosine

Sulfamoyl chloride (**1**): Chlorosulfamoyl isocyanate (3.10 g, 21.9 mmol) was placed in a dry two-necked flask equipped with a $CaCl_2$ drying tube. Formic acid (1.00 g, 21.7 mmol) was added dropwise via syringe to the flask with ice cooling. Vigorous reaction occurred, and effervescence was observed. After the addition of HCOOH, the mixture was stirred at room temperature until no more gas was evolved. After stirring at room temperature for 1 h, colorless crystals were accumulated in the flask. Benzene (10 ml) was added to the reaction mixture, and insoluble material was removed by filtration. The solution was evaporated to give **1** as colorless crystals (1.86 g, 73%).

2′,3′-O-Isopropylidene-5′-O-sulfamoyladenosine (**2**): To a suspension of NaH (0.174 g, 55% in paraffin, 3.94 mmol, washed with *n*-hexane) in 1,4-dioxane (50 ml) was added 2′,3′-*O*-isopropylideneadenosine (0.806 g, 2.62 mmol) at 0 °C under a nitrogen atmosphere. After stirring for 30 min at 0 °C, a solution of **1** (0.481 g, 4.16 mmol) in 1,4-dioxane (5 ml) was added dropwise to the suspension at 0 °C. The mixture was stirred at 0 °C for 30 min, and ice bath was removed. The mixture was stirred at room temperature for 24 h. Anhydrous methanol (20 ml) was added to the reaction mixture, and the resulting mixture was filtered through Celite pad. The filtrate was evaporated to dryness, and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 9:1) to give **2** as a colorless crystals (0.87 g, 86%): IR (KBr) 3354, 3188, 2943, 1651, 1601, 1576, 1373, 1182, 1074, 858, 799 and 556 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.34 (s, 3H, CH_3), 1.55 (s, 3H, CH_3), 4.13 (dd, 1H, J = 10.5 and 6.3 Hz, ribose H-5′a), 4.24 (dd, 1H, J = 10.5 and 5.3 Hz, ribose H-5′b), 4.40 (m, 1H, ribose H-4′), 5.08 (dd, 1H, J = 6.3 and 3.3 Hz, ribose H-3′), 5.43 (dd, 1H, J = 6.3 and 2.3 Hz, ribose H-2′), 6.23 (d, 1H, J = 2.3, ribose H-1′), 7.38 (br s, 2H, adenine NH_2), 7.61 (br s, 2H, SO_2NH_2), 8.17 (s, 1H, adenine H-8), 8.30 (s, 1H, adenine H-2); ^{13}C NMR (DMSO-d_6) δ: 25.58 (CH_3), 27.37 (CH_3), 68.57 (ribose C-5′), 81.64 (ribose C-3′), 83.81 (ribose C-2′), 84.10 (ribose C-4′), 89.59 (ribose C-1′), 114.07 (C), 119.56 (adenine C-5), 140.32 (adenine C-8), 149.20 (adenine C-4), 153.34 (adenine C-2), 156.62 (adenine C-6).

Ketoprofen N-hydroxysuccinimide ester (**3**): 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (0.929 g. 4.85 mmol) and *N*-hydroxysuccinimide (0.570 g, 4.96 mmol) was dissolved in 1,4-dioxane (5 ml) under a nitrogen atmosphere. After stirring for 10 min at room temperature, a solution of ketoprofen (0.827 g, 3.25 mmol) in 1,4-dioxane (15 ml) was added dropwise to the solution at room temperature. The mixture was stirred at room temperature for 24 h. The reaction mixture was poured into $CHCl_3$ and washed successively with 1 N HCl, sat. $NaHCO_3$ and sat. NaCl, and dried over Na_2SO_4. The organic layer was evaporated to dryness, and the residue was purified by silica gel column chromatography (hexane/EtOAc/acetic acid, 4:6:1.2%) to give **3** as an colorless oil (0.961 g, 84%): IR (KBr) 1811, 1782, 1736, 1647, 1597, 1283, 1202, 1119, 1063, 986, 964, 856, 714 and 702 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.66 (d, 3H, J = 7.1, CH_3), 2.81 (s, 4H, CH_2CH_2), 4.12 (q, 1H, J = 7.1, CH), 7.47–7.83 (m, 9H, 3-benzoylphenyl); ^{13}C NMR ($CDCl_3$) δ: 18.91 (CH_3), 25.62 (CH_2CH_2), 42.83 (C), 128.44, 129.00, 129.30, 129.69, 130.23, 131.61, 132.67, 137.28, 138.20, 138.53 ($C_6H_5COC_6H_4$), 169.53 (CO), 196.36 (CO).

2′,3′-O-Isopropilidene-5′-O-(N-ketoprofenylsulfamoyl)adenosine (**4**): A solution of **2** (0.692 g, 1.79 mmol) and **3** (0.943 g, 2.68 mmol) in acetonitrile (50 ml) was cooled at 0 °C, and cesium carbonate (0.705 g, 2.16 mmol) was added under a nitrogen atmosphere. The mixture was stirred at 0 °C for 1 h, and the ice bath was removed. The mixture was stirred at room temperature for 19 h. The reaction mixture was evaporated to dryness, and the residue was purified by open column chromatography on Chromatorex NH to give **4** as a colorless crystals (0.781 g, 70%): R_f = 0.66 (Merk TLC, EtOAc:2-butanone:EtOH:H_2O = 5:3:1:1, anisaldehyde), R_f = 0.57 (NH-TLC, H_2O:MeCN = 1:5): IR (KBr) 3341, 3202, 2936, 1649, 1576, 1475, 1375, 1288, 1148, 1076, 1001, 845, 777, 721 and 644 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.24 (s, 1.5H, CH_3, diastereomer), 1.25 (s, 1.5H, CH_3, diastereomer), 1.27 (d, 1.5H, J = 7.0, CH_3, diastereomer), 1.28 (d, 1.5H, J = 7.0, CH_3, diastereomer), 1.49 (s, 3H, CH_3), 3.49 (q, 1H, J = 7.0, CH), 3.89 (m, 2H, ribose H-5′), 4.32 (m, 1H, ribose H-4′), 4.90 (m, 1H, ribose H-3′), 5.25 (dd, 0.5H, J = 6.0 and 3.0, ribose H-2′, diastereomer), 5.28 (dd, 0.5H, J = 6.0 and 3.0, ribose H-2′, diastereomer), 6.10 (d, 1H, J = 3.0, ribose H-1′), 7.33 (br s, 2H, adenine NH_2), 7.38–7.72 (m, 9H, 3-benzoylphenyl), 8.12(s, 0.5H, adenine H-8, diastereomer), 8.13 (s, 0.5H, adenine H-8, diastereomer), 8.35(s, 0.5H, adenine H-2, diastereomer), 8.36 (s, 0.5H, adenine H-2, diastereomer); ^{13}C NMR (DMSO-d_6) δ: 19.67 (CH_3), 25.55 (CH_3), 27.45 (CH_3), 49.22 (C), 67.60 (ribose C-5′), 82.03 (ribose C-3′), 83.96 (ribose C-2′), 84.24 (ribose C-4′), 89.71 (ribose C-1′), 113.62 (C), 119.30 (adenine C-5), 128.01, 128.61, 129.00, 129.27, 130.14, 132.50, 133.04, 137.03, 137.56, 145.02

($C_6H_5COC_6H_4$), 140.00 (adenine C-8), 149.49 (adenine C-4), 153.28 (adenine C-2), 156.56 (adenine C-6), 179.05 (CO), 196.35 (CO).

5′-O-(N-Ketoprofenylsulfamoyl)adenosine (**5**): Compound **4** (0.108 g, 0.173 mmol) was dissolved in anhydrous methanol (3.8 ml). Conc. HCl (0.2 ml) was added to the solution, and the mixture was stirred for 1 h at 60 °C. The pH of the reaction mixture was adjusted to 8.5 by adding 28% NH_3 aq at 0 °C. The resulting mixture was evaporated to dryness, and the residue was purified by open column chromatography on Chromatorex NH to give **5** as a white solid (0.101 g). By comparison with the ^{1}H NMR integration value of internal standard, 3-trimethylsilyl-1-propanesulfonic acid, the purity was determined as 46%: R_f = 0.43 (Merk TLC, EtOAc:2-butanone:EtOH:H_2O = 5:3:1:1, anisaldehyde), R_f = 0.29 (NH-TLC, H_2O:MeCN = 1:5): $[M-H]^{-1}$ observed 581.1453, predicted 581.1455; IR (KBr) 3352, 3211, 2974, 1651, 1599, 1479, 1288, 1148, 999, 849, 777, 721, 644 and cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 1.27 (d, 1.5H, CH_3, diastereomer), 1.28 (d, 1.5H, CH_3, diastereomer), 3.49 (m, 1H, diastereomer), 3.92 (m, 1H, ribose H-5′a), 3.98 (m, 1H, ribose H-5′b), 4.03 (m, 1H, ribose H-4′), 4.10 (m, 1H, ribose H-3′), 4.57 (m, 1H, ribose H-2′), 5.29 (d, 1H, OH), 5.47 (d, 1H, OH), 5.88 (d, 1H, ribose H-1′), 7.28 (br s, 2H, adenine NH_2), 7.40–7.73 (m, 9H, 3-benzoylphenyl), 8.12 (s, 1H, adenine H-8), 8.38 (s, 1H, adenine H-2); ^{1}H NMR (D_2O) δ: 1.40 (d, 1H, CH_3), 4.04–4.39 (m, 5H, ribose H-5′, H-4′, H-3′, H-2′), 5.81 (d, 0.5H, ribose H-1′, diastereomer), 5.78 (d, 0.5H, ribose H-1′, diastereomer), 7.14–7.59 (m, 11H, 3-benzoylphenyl and adenine NH_2), 7.96 (s, 0.5H, diastereomer), 7.97 (s, 0.5H, diastereomer), 8.02 (s, 0.5H, diastereomer), 8.04 (s, 0.5H, diastereomer); ^{13}C NMR (DMSO-d_6) δ: 19.86 (CH_3), 49.17 (C), 67.74 (ribose C-5′), 71.28 (ribose C-3′), 74.10 (ribose C-2′), 83.02 (ribose C-4′), 87.52 (ribose C-1′), 119.38 (adenine C-5), 128.02, 128.67, 129.04, 129.33, 130.15, 132.56, 133.07, 137.04, 137.56, 145.08 ($C_6H_5COC_6H_4$), 139.93 (adenine C-8), 150.05 (adenine C-4), 153.17 (adenine C-2), 156.47 (adenine C-6), 179.00 (CO), 196.45 (CO).

2.3. Plasmid construction and expression of LUC-H protein

The LUC-H gene was amplified from pHLfLK by PCR with forward (TTTAA*CATATG*GAAAACATGGAGAACG) and reverse (TGATTAAAC*TCTAGA*TTGACATTTACATC) primers. These primers were designed with *Nde* I and *Xba* I sites (italicized) at the 5′ and 3′ ends of the LUC-H gene, respectively. The underlined ATG represents the start codon. The amplified 1.7 kbp fragment was subcloned to a TA vector, which was prepared from pXCmkn12 digested with *Xcm* I (National Institute of Genetics, Japan). The subcloned vector was first digested with *Nde* I and *Xba* I, then purified and inserted into a pCold I expression vector (Takara, Japan). The resultant plasmid, pColdI-LUC-H, was used to express the recombinant LUC-H protein. *Escherichia coli* BL21(DE3) cells were transformed with pColdI-LUC-H, cultured, and induced to express LUC-H according to manufacturer's instructions. After production of the recombinant enzyme, the cells were collected, disrupted by sonication (20 kHz, 30 s × 10 times) in 5 ml of 50 mM potassium phosphate buffer (PPB) (pH 7.0) containing 300 mM NaCl, and centrifuged (14,500 × *g* for 10 min, 4 °C). The enzyme was purified from the supernatant using TALON Metal Affinity Resin (2 ml) (Clontech) according to manufacturer's instructions and identified by SDS–PAGE analysis. The fractions containing LUC-H were combined and dialyzed overnight against 100 mM Tris–HCl buffer (pH 7.5).

2.4. Spectrophotometric assay for LUC-H activity

The activity of LUC-H was determined by UV–vis spectrophotometry. In this assay, we measured the initial rate of AMP formation by coupling the reaction of LUC-H with adenylate kinase, pyruvate kinase, and lactate dehydrogenase and following NADH oxidation at 340 nm (6220 M^{-1} cm^{-1}) with a spectrophotometer (U-2800A, Hitachi) [15–17]. The standard reaction mixture for this assay contained 100 mM PPB (pH 7.0), 10 mM ATP, 10 mM $MgCl_2$, 0.25 mM (*R*)-ketoprofen, 2 mM CoASH, 1 mM phosphoenolpyruvic acid, 0.2 mM NADH, 40 mg/ml adenylate kinase, 20 mg/ml pyruvate kinase, 20 mg/ml lactate dehydrogenase, and 100 mg of LUC-H. The total volume was brought to 500 μl. The mixture containing all components except LUC-H was incubated at room temperature (27 ± 2 °C) for 3 min. The reaction was then initiated by addition of the enzyme.

2.5. Kinetic analysis and determination of K_i value

The kinetic parameters were determined by spectrophotometry. The k_{cat} and K_m values were evaluated by Michaelis–Menten analysis using GraphPad Prism, version 5.01 (GraphPad, USA). The k_{cat} values were expressed as the turnover numbers per subunit (Mr of the subunit, 60,163). The kinetic studies were performed with varying concentrations of the test substrate while the concentrations of all other components were fixed. The default concentrations of the three cofactors, ketoprofen, ATP, and CoASH, were 0.25 mM, 10 mM, and 2 mM respectively. The concentrations of substrate for measuring each parameter were 0.05–1 mM ((*R*)- or (*S*)-ketoprofen), 0.1–2.5 mM (ATP), and 0.1–3 mM (CoASH) respectively. Each assay was repeated five times.

Inhibition studies were performed with a ketoprofenyl-AMP intermediate analogue. Concentrations of the analogue were 0.05 and 0.1 mM. Each set of assays was performed five times. The K_i value was determined by fitting initial velocity data obtained by the spectrophotometric method described above with the appropriate rate equation in GraphPad Prism.

3. Results and discussion

3.1. Substrate specificity of recombinant LUC-H

The specific activity of recombinant LUC-H was measured using a spectrophotometric method (Table 1). LUC-H exhibited thioesterification activity toward a series of 2-arylpropanoic acids, including flurbiprofen, ibuprofen, ketoprofen, and naproxen. Among these 2-arylpropanoic acids, ketoprofen was the best substrate. When the concentration of (±)-ketoprofen was 0.25 mM, the specific activity of LUC-H was 20.5 nmol/min/mg, which was comparable to the previously reported value obtained with an HPLC assay [11]. The same spectrophotometric method was used to analyze substrate specificity. 2-Phenylbutanoic acid was accepted by LUC-H, although the specific activity was very low. When 2-phenylpentanoic acid, 2-phenyl-3-methylbutanoic acid, 2-(4-chlorophenoxy)propanoic acid, and 2-methyl-3-phenylpropanoic acid were used, the catalytic activity of LUC-H was below the limit of detection of the assay. Fatty acids with various chain lengths were also tested with this assay. As shown in Table 1, LUC-H exhibited thioesterification activity only toward fatty acids with 8–18 carbons and the highest activity was detected with dodecanoic acid (C12). These results indicate that the substrate specificity of LUC-H toward fatty acids is similar to that of the LACS family. 6-Mercaptohexanoic acid and 11-mercaptoundecanoic acid, both of which have a thiol group at the ω-position, were also converted to the corresponding coenzyme A thioesters. 6-Aminohexanoic acid was not converted to a coenzyme A thioester; however, the catalytic activity of LUC-H recovered when the ω-amino group was protected by a *tert*-butoxycarbonyl (Boc) or benzyloxycarbonyl (Cbz) group. This result indicates that the identity of substituents at the ω-position of the fatty acid affects the substrate accessibility of this enzyme. Benzoic acid, 2-anthracenecarboxylic acid, anthraquinone-2-carboxylic acid, and

Table 1
Substrate specificity of the LUC-H catalyzed thioesterification reaction.

Carboxylic acid[a]	Relative activity[b] (%)
(±)-Flurbiprofen	10
(±)-Ibuprofen	8
(±)-Ketoprofen	46
(±)-Naproxen	15
(±)-Phenylbutanoic acid	4
(±)-2-Phenylpentanoic acid	N.D.
(±)-2-Phenyl-3-methylbutanoic acid	N.D.
(±)-2-(4-Chlorophenoxy)propanoic acid	N.D.
(±)-2-Methyl-3-phenylpropanoic acid	N.D.
Acetic acid (C2)	N.D.
Propanoic acid (C3)	N.D.
Butanoic acid (C4)	N.D.
Hexanoic acid (C6)	N.D.
Octanoic acid (C8)	7
Decanoic acid (C10)	93
Dodecanoic acid (C12)	100
Butyldecanoic acid (C14)[c]	64
Hexadecanoic acid (C16)[c]	3
Octadecanoic acid (C18)[c]	3
6-Mercaptohexanoic acid	16
11-Mercaptoundecanoic acid	99
6-Aminohexanoic acid (Ahx)	N.D.
Boc-Ahx	58
Cbz-Ahx	23
Benzoic acid	32
2-Anthracenecarboxylic acid	12
Anthroquinone-2-carboxylic acid	49
1-Pyrenecarboxylic acid	13
Ferrocenecarboxylic acid	N.D.
Phenylglycine	N.D.
Mandelic acid	N.D.
Dihydrocinnamic acid	N.D.
trans-Cinnamic acid	N.D.
Malonic acid	N.D.
Retionic acid	N.D.
α-Lipoic acid	N.D.

N.D. = not detected; the relative activity could not be calculated because the specific activity was below the detection limit of the assay.

[a] Final concentration of carboxylic acid was 0.25 mM.

[b] The relative activity was calculated by comparison with the specific activity, using the value for dodecanoic acid (C12) (44.6 nmol/min/mg) as a standard. The specific activity of thioester formation was determined using the spectrophotometric assay.

[c] 0.2% Triton X-100 was added to the reaction mixture for this assay to ensure complete dissolution of the substrate.

1-pyrenecarboxylic acid were also converted to the corresponding thioesters in moderate efficiency. These results imply that LUC-H can accept bulky substitutions at the α-position of the carboxyl group. Ferrocenecarboxylic acid, phenylglycine, mandelic acid, dihydrocinnamic acid, *trans*-cinnamic acid, malonic acid, retionic acid, and α-lipoic acid were not accepted by this enzyme. It is interesting that α-lipoic acid was not accepted as a thioesterification substrate because Niwa et al. concluded that α-lipoic acid acts as a competitive inhibitor of the bioluminescence reaction rather than being converted into a thioester [18]. Our result supports their conclusion and adds to knowledge of the inhibitory pattern of this compound.

3.2. Determination of kinetic parameters of LUC-H catalyzed enantioselective thioesterification of ketoprofen

To obtain information about the enantiodifferentiation mechanism of the LUC-H catalyzed thioesterification reaction, we determined its kinetic parameters using ketoprofen as the carboxylic acid substrate. It has been proposed that the firefly luciferase catalyzed thioesterification reaction occurs in two steps (Fig. 2) [7,11]. First, the carboxylic acid substrate couples with ATP to generate an acyl-AMP intermediate. Then CoASH attacks

Table 2
Kinetic parameters of the LUC-H catalyzed thioesterification reaction.

Substrate	Ketoprofen			ATP			CoASH		
	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
(*R*)-Ketoprofen	0.16 ± 0.02	0.17 ± 0.009	1.06	0.52 ± 0.06	0.077 ± 0.003	0.15	0.44 ± 0.03	0.081 ± 0.002	0.18
(*S*)-Ketoprofen	0.06 ± 0.01	0.002 ± 0.00007	0.04	0.50 ± 0.06	0.0006 ± 0.00003	0.013	0.86 ± 0.14	0.0003 ± 0.00002	0.0004

The specific activity of thioester formation was determined using the spectrophotometric assay.

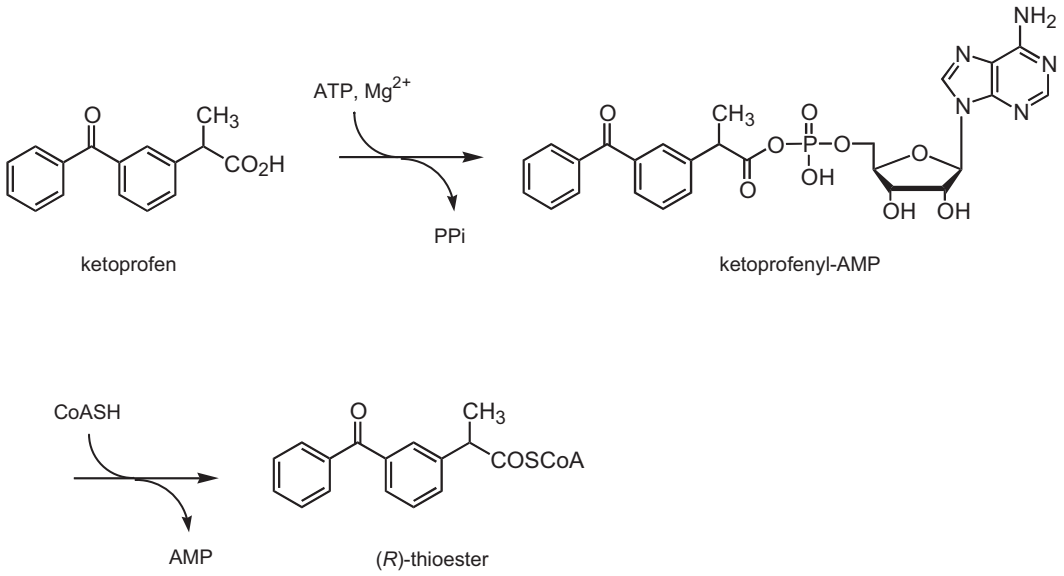

Fig. 2. Chemical conversion process of the firefly luciferase catalyzed thioesterification reaction.

this intermediate, releasing the thioester product. LUC-H can distinguish the absolute configuration of ketoprofen in either of these steps, and the (*R*)-acid is preferentially converted to the thioester about 20 times faster than the (*S*)-antipode [11]. Three substrates, ketoprofen, ATP, and CoASH, take part in this transformation process. Thus the affinity and/or catalytic rate of these compounds must be different when ketoprofen enantiomer is subjected separately to the reaction. We therefore determined the kinetic parameters of each of these three compounds (Table 2). The K_m values of the ketoprofen enantiomers show that the affinity of (*S*)-ketoprofen, the less reactive substrate, is two fold less than that of (*R*)-ketoprofen, the more reactive substrate. The affinities

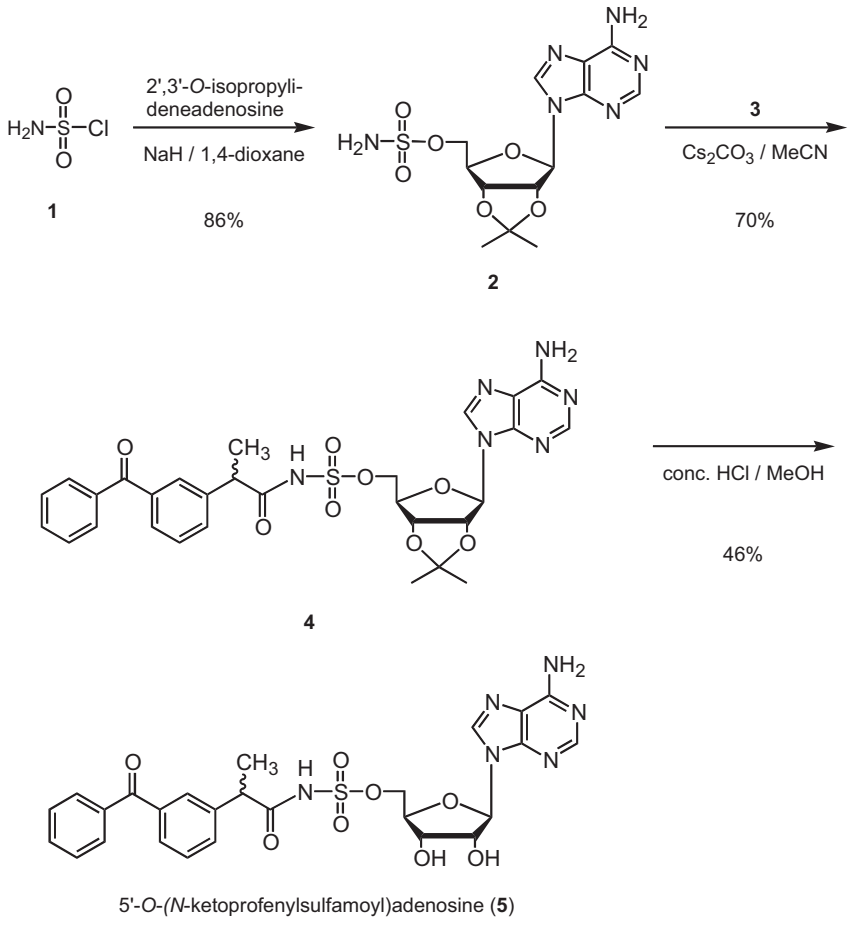

Fig. 3. Synthesis of the ketoprofenyl-AMP intermediate analogue, 5′-*O*-(*N*-ketoprofenylsulfamoyl)adenosine (**5**).

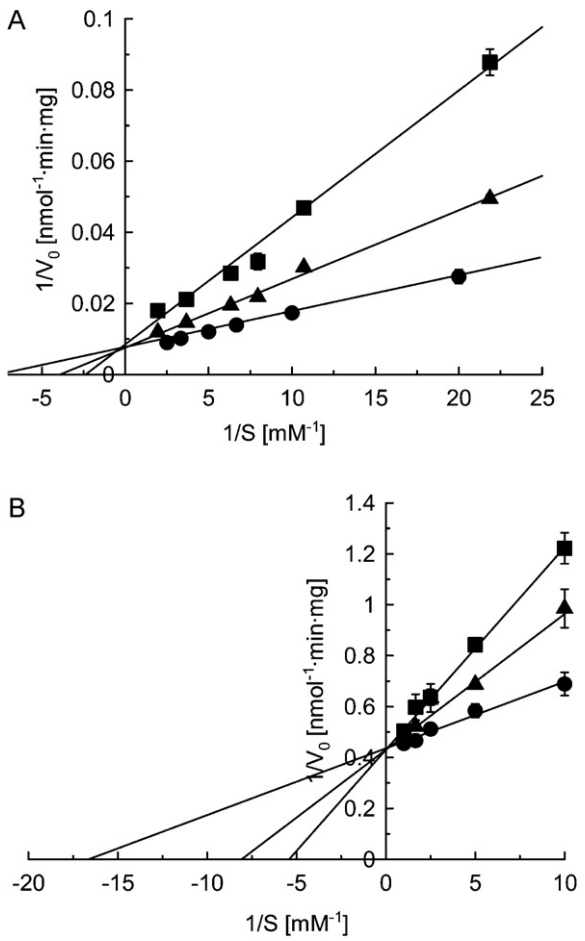

Fig. 4. Inhibition of LUC-H by a ketoprofenyl-AMP intermediate analogue with respect to (*R*)-ketoprofen (A) and (*S*)-ketoprofen (B). The concentrations of the analogue compound used were 0 (circle), 0.05 mM (triangle), 0.1 mM (square), respectively.

of the ATP and CoASH cofactors for LUC-H are independent of the absolute configuration of the ketoprofen substrate. These results suggest that all components of the reaction have an equal chance to bind to the active site of enzyme with respect to the enantiomers of the ketoprofen substrate before the enantiodifferentiation process occurs. In contrast, the k_{cat} values show an obvious difference between the optical isomers of ketoprofen. The value of k_{cat} for (*R*)-ketoprofen is approximately 100 times larger than that observed for (*S*)-ketoprofen. Because of this difference, the product from the (*R*)-enantiomer dominates the reaction outcome.

3.3. Inhibitory effect of an acyl-AMP intermediate analogue

The kinetic parameters indicated that there are no differences in the affinities for all substrates regardless of the chirality of ketoprofen. Next, we focused our attention on the acyl-AMP intermediate and investigated the inhibitory effect of an analogue on the enzyme. We used the *N*-acyl sulfamate compound 5′-*O*-(*N*-ketoprofenylsulfamoyl)adenosine (**5**), a stable analogue of the intermediate ketoprofenyl-AMP (Fig. 3). We expected this compound to function as an inhibitor of thioester formation because a similar compound was used for enzyme-analogue structural analysis of *L. cruciata* firefly luciferase [19]. The synthesis was undertaken with commercially available 2′,3′-*O*-isopropylideneadenosine and this compound was converted into 2′,3′-*O*-isopropylidene-5′-*O*-sulfamoyladenosine (**2**) by reaction with sulfamoyl chloride (**1**). By cesium carbonate mediated condensation of **2** and **3**, 2′,3′-*O*-isopropilidene-5′-*O*-(*N*-ketoprofenylsulfamoyl)adenosine (**4**) was obtained in good yield and the isopropylidene moiety was easily deprotected under acidic condition to obtain target analogue (**5**). In the presence of this analogue, we measured the initial rate of the thioesterification reaction of (*R*)- or (*S*)-ketoprofen and compared the patterns of inhibition. We found that the analogue compound behaved as a competitive inhibitor with K_i values of 0.059 and 0.064 mM for (*R*)- and (*S*)-ketoprofen, respectively (Fig. 4). These results indicate that both enantiomers of ketoprofen could bind to the active site of LUC-H and be converted to the acyl-AMP intermediate with similar reaction efficiency. The results of the kinetic parameter determination demonstrated that the affinity of all components of the reaction is independent of the chirality of ketoprofen. We therefore concluded that the first adenylation step is not enantioselective, and the enantiodifferentiation is achieved in the second thioester-forming step.

Our results are consistent with the action of firefly luciferase on the luciferin substrate, which behaves differently after the formation of the luciferyl-AMP intermediate, depending on the chirality of luciferin [4–6]. When D-luciferin is the substrate, the oxidation reaction occurs and produces bioluminescence, whereas when L-luciferin is the substrate, the thioesterification reaction proceeds in the presence of CoASH. A domain movement mechanism for acyladenylate-forming enzymes has been proposed, based on available structural and functional information [20–23]. In the case of the firefly luciferase-catalyzed bioluminescent reaction, there is some evidence that movement of the C-terminal domain is necessary for light production [24,25]. Therefore, in the case of the thioesterification reaction of ketoprofen, it is not inconsistent that a similar movement would occur after the formation of ketoprofenyl-AMP intermediate. Different movement of the C-terminal domain depending on substrate chirality may cause the difference in the k_{cat} value that results in enantiodifferentiation by this enzyme.

4. Conclusions

We have analyzed the substrate specificity of recombinant LUC-H using a spectrophotometric assay system to easily judge the reaction efficiency. We also determined the kinetic parameters of the enantioselective thioesterification reaction of ketoprofen. Based on the kinetic parameters, we concluded that a difference in k_{cat} between (*R*)-ketoprofen and (*S*)-ketoprofen contributes significantly to the enantiodifferentiation event. The results of our inhibition experiment suggest that the enantiodifferentiation event occurs after the formation of the acyl-AMP intermediate.

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