Journal of Molecular Catalysis B: Enzymatic 102 (2014) 115–119

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Efficient preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid catalyzed by *Shinella* sp. R-6 and *Arthrobacter* sp. S-2

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ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 25 January 2014
Accepted 29 January 2014
Available online 14 February 2014

Keywords:
Amidase
Enrichment culture technique
Kinetic resolution
Screening
Trifluoromethyl

ABSTRACT

Several microorganisms that can enantioselectively hydrolyze 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide have been isolated from soil samples. These strains were capable of growing in a medium containing 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide as the sole nitrogen source. Among them, *Shinella* sp. R-6 was identified as a strain capable of exhibiting *R*-selective hydrolysis activity, while *Arthrobacter* sp. S-2 was capable of exhibiting *S*-selective hydrolysis activity. The preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid *via* the two-step whole-cell reaction was investigated using these two strains.

1. Introduction

Trifluoromethyl-substituted compounds, especially optically active ones, have received much attention of scientists and industry [1]. Both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid **1**, containing hydroxyl and trifluoromethyl groups attached to the same carbon atom, were previously shown to be intermediates for the synthesis of a number of fine chemicals and pharmaceuticals, like the pyruvate dehydrogenase kinase inhibitor for the treatment of diabetes, and bradykinin antagonist in anti-inflammatory and painkiller medicines (Fig. 1) [2,3].

Many microbial amidases have been characterized and applied to the synthesis of useful compounds for the preparation of carboxylic acids [4]. Shaw has reported a process for the large-scale synthesis of enantiomerically pure acid **1**, which employed an enantioselective amidase from *Klebsiella oxytoca* PRS1 and used it in the kinetic resolution of (±)-amide **2** to obtain (*R*)-acid **1** and (*S*)-amide **2** [5]. While this enzymatic procedure is a very efficient way, there still remains a drawback to this method. To obtain (*S*)-acid **1**, the recovered (*S*)-amide **2** has to be hydrolyzed under harsh conditions. Konigsberger has reported the process that employed the enzymatic resolution of 1,1,1-trifluoroacetone cyanohydrin acyl derivatives followed by the hydrolysis to yield (*R*)-acid **1** [6]. However, this method showed low enantioselectivity for (*S*)-acid **1**. Therefore, the efficient preparation of both enantiomers of this valuable molecule is still desired.

In this study, we focused on enantioselective hydrolysis activity from microbial sources that acted on amide **2** to develop kinetic resolution for the preparation of both enantiomers of acid **1** under mild conditions.

2. Materials and methods

2.1. Materials

(±)-Amide **2** was prepared by the hydrolysis of the corresponding cyanohydrin prepared from 1,1,1-trifluoroacetone and NaCN according to a previously reported procedure [5,7]. All other chemicals were purchased from commercial sources and were used without further purification.

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http://dx.doi.org/10.1016/j.molcatb.2014.01.024
1381-1177/

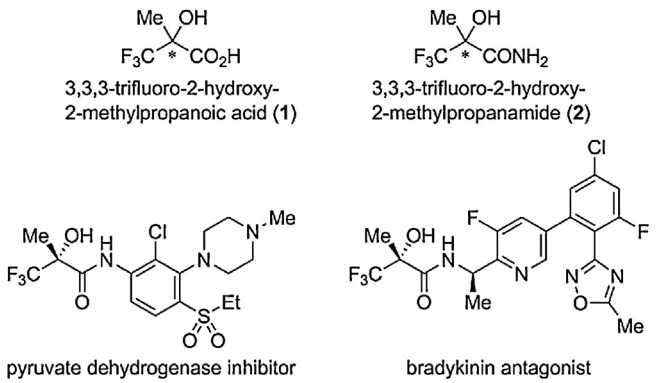


Fig. 1. 3,3,3-Trifluoro-2-hydroxy-2-methylpropanoic acid **1** and the corresponding amide **2**, as intermediates for the synthesis of pharmaceuticals.

2.2. Isolation of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide (**2**) degrading microorganisms using the enrichment culture technique

A screening medium consisting of 2.0 g of KH_2PO_4, 2.0 g of K_2HPO_4, 1.0 g of NaCl, 0.50 g of yeast extract, 0.20 g of $MgSO_4$, 1.0 mL of the vitamin mixture solution, 1.0 mL of the trace element solution, and 2.0 g of (±)-amide **2** in 1000 mL of water was used to isolate amide **2** degrading microorganisms. The vitamin mixture solution was composed of 20 mg of inositol, 4.0 mg of nicotinic acid, 4.0 mg of Ca·pantothenate, 4.0 mg pyridoxine·HCl, 4.0 mg of thiamine·HCl, 2.0 g of *p*-aminobenzoic acid, 2.0 mg of riboflavin, 0.10 mg of folic acid, and 20 μg of biotin in 100 mL of water. The trace element solution was composed of 500 mg of CyDTA, 200 mg of $FeSO_4{\cdot}7H_2O$, 30 mg of H_3BO_4, 20 mg of $CoCl_2{\cdot}6H_2O$, 10 mg of $ZnSO_4{\cdot}7H_2O$, 3.0 mg of $MnCl_2{\cdot}4H_2O$, 3.0 mg of Na_2MoO_4, 2.0 mg of $NiCl_2{\cdot}6H_2O$, and 1.0 mg of $CuCl_2{\cdot}2H_2O$ in 100 mL of water. Amide **2** degrading microorganisms were isolated as follows. Soil samples were taken from different locations in Toyama, Japan. A spoonful of the soil sample was added to 2.5 mL of screening medium, and incubated with reciprocal shaking (300 strokes/min) at 30 °C. A 50 μL aliquot was transferred to fresh medium every 3 or 4 days. After three transfers, each of the culture broths was spread onto agar plates containing the same medium and 1.5% agar, respectively.

2.3. Screening and analytical method

The medium used for screening was the same as that described above. Microorganisms isolated from soil samples described above or stored as stock cultures were inoculated into 2.5 mL of medium, and then incubated with reciprocal shaking (300 strokes/min) for 48 h at 30 °C. Cells were harvested by centrifugation (20,000 × *g*, 5 min, 4 °C) and washed with 20 mM potassium phosphate buffer (KPB, pH 7.0). Wet cells from 2.0 mL of the culture were suspended in 980 μL of 20 mM KPB (pH 7.0). A total of 20 μL of 10% (±)-**2** aqueous solution was added to this suspension as a substrate and the mixture was incubated with shaking at 1000 rpm for 18 h at 30 °C. A 600 μL aliquot was withdrawn after centrifugation (20,000 × *g*, 10 min, 4 °C) and mixed with 400 μL of water. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed using HPLC with the Cosmosil® C_{18}-MS-II column (5 μm, 4.6 mm × 150 mm, Nacalai Tesque, Inc.) (solvent: 0.1% H_3PO_4 aq./MeCN = 9/1; flow rate: 0.80 mL/min; temperature: 40 °C; detection: 210 nm; t_R = 4.7 min for amide **2** and 7.6 min for acid **1**) for conversion and with the Sumichiral OA-5000 column (5 μm, 4.6 mm × 150 mm, Sumika Chemical Analysis Service, Ltd.) [solvent: 2 mM $CuSO_4$ aq./MeCN = 85/15; flow rate: 2.0 mL/min; temperature: 40 °C; detection: 254 nm; t_R = 13.6 min for (*R*)-**1** and 16.0 min for (*S*)-**1**] for enantiomeric excess.

2.4. Examination of substrate concentration

Shinella sp. R-6 or *Arthrobacter* sp. S-2 cells were inoculated into 5.0 mL of the medium as described above, and incubated with shaking at reciprocal shaking (300 strokes/min) for 24 h at 30 °C. Cells were harvested by centrifugation (20,000 × *g*, 10 min, 4 °C) and washed with 20 mM KPB (pH 7.0). Wet cells from 10 mL of the culture were suspended in 20 mM KPB (pH 7.0) containing 10–1000 mg of (±)-**2** (sub. conc. 0.20–20%) in a total volume of 5.0 mL. A 1000 μL aliquot was withdrawn after incubation with reciprocal shaking (300 strokes/min) for an appropriate period at 30 °C. After appropriate dilution and centrifugation (20,000 × *g*, 10 min, 4 °C), an 800 μL of supernatant was then withdrawn. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed by HPLC, as described above.

2.5. Kinetic resolution of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide (**2**) by the two-step reaction using Shinella sp. R-6 and Arthrobacter sp. S-2

Arthrobacter sp. S-2 cells were inoculated into 5.0 mL of the medium as described above, and incubated with reciprocal shaking (300 strokes/min) for 24 h at 30 °C. Grown cells were added into 500 mL of the same medium, and incubated with shaking at 150 rpm for 24 h at 30 °C. A 200 mL aliquot was withdrawn, and cells were harvested by centrifugation (9000 × *g*, 10 min, 4 °C) and washed with 20 mM KPB (pH 7.0). Wet cells from 200 mL of the culture were suspended in 20 mM KPB (pH 7.0) containing 2.00 g of (±)-**2** (sub. conc. 2.0%) in a total volume of 100 mL. A 1000 μL aliquot was withdrawn after incubation for an appropriate period at 30 °C with shaking at 150 rpm. After centrifugation (20,000 × *g*, 10 min, 4 °C), an 800 μL of supernatant was withdrawn. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed by HPLC with the Cosmosil® C_{18}-MS-II column as described above. After incubation for 22 h at 30 °C with shaking at 150 rpm, the reaction mixture was centrifuged (9000 × *g*, 10 min, 4 °C). The supernatant was collected, and wet cells were washed with water (10 mL) twice. The combined supernatant was extracted with EtOAc five times. The combined organic extract was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was the recovered (*R*)-**2** (991 mg, 50%). The residual aqueous layer was acidified to pH 4 by the addition of 2 N HCl, and extracted with Et_2O five times. The combined organic extract was dried over $MgSO_4$, and concentrated *in vacuo*. The residue was (*S*)-**1** (921 mg, 46%). Based on HPLC analysis with the Sumichiral OA-5000 column as described above, the enantiomeric excess of (*S*)-**1** was determined to be 96.7%.

Shinella sp. R-6 cells were inoculated into 5.0 mL of the medium as described above, and incubated with reciprocal shaking (300 strokes/min) for 24 h at 30 °C. Grown cells were added into 500 mL of the same medium, and incubated with shaking at 150 rpm for 48 h at 30 °C. A 400 mL aliquot was withdrawn, and cells were harvested by centrifugation (9000 × *g*, 10 min, 4 °C) and washed with 20 mM KPB (pH 7.0). Wet cells from 400 mL of the culture were suspended in 20 mM KPB (pH 7.0) containing the recovered (*R*)-**2** described above (991 mg) in a total volume of 200 mL. A 1000 μL aliquot was withdrawn after incubation for an appropriate period at 30 °C with shaking at 150 rpm. After centrifugation (20,000 × *g*, 10 min, 4 °C), an 800 μL of supernatant was withdrawn. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed by HPLC with the Cosmosil® C_{18}-MS-II column as described above. After incubation for 34 h at 30 °C with shaking at 150 rpm, the reaction mixture was centrifuged (9000 × *g*, 10 min, 4 °C). The supernatant was collected, and wet cells were washed

Table 1
3,3,3-Trifluoro-2-hydroxy-2-methylpropanamide **2** hydrolysis activity from stock cultures.

Genus	Number of strains			
	Microorganisms tested	Hydrolysis of **2** (>10%)	Stereoselectivity toward **2** (>50% ee of **1**)	
			R	*S*
Pseudomonas	78	14	3	2
Arthrobacter	51	7	–	5
Alcaligenes	34	6	–	4
Brevibacterium	10	3	2	–
Variovorax	8	3	1	–
Corynebacteirum	18	2	–	–
Yersinia	3	2	1	–
Microbacterium	9	2	–	1
Klebsiella	8	1	–	1
Rhodococcus	16	1	–	–
Serratia	10	1	–	1
Acinetobacter	4	1	1	–
Others	193	–	–	–
Total	442	43	8	14

with water (10 mL) twice. The combined supernatant was washed with EtOAc five times, acidified to pH 4 by the addition of 2 N HCl, and extracted with Et_2O five times. The combined organic extract was dried over $MgSO_4$, and concentrated *in vacuo*. The residue was (*R*)-**1** (902 mg, 90%, and 45% for two steps). Based on HPLC analysis with the Sumichiral OA-5000 column as described above, the enantiomeric excess of (*R*)-**1** was determined to be 99.5%.

3. Results

3.1. Isolation and identification of microorganisms

We have screened microorganisms that can enantioselectively hydrolyze (±)-amide **2** from stock cultures. After growing on the screening medium containing 0.20% (±)-amide **2** as the sole nitrogen source, more than 400 microorganisms from 19 genera were assayed for hydrolytic activity on (±)-amide **2** (Table 1). Although some microorganisms were obtained that could show hydrolytic activity on amide **2**, most of them showed no or scarce enantioselectivity.

We have also screened microorganisms that can assimilate (±)-amide **2** as the sole nitrogen source. After growing on the screening medium containing 0.20% (±)-amide **2** from 50 soil samples, the 188 isolated microorganisms were assayed for hydrolytic activity on (±)-amide **2** (Table 2). Although some microorganisms were obtained that exhibited hydrolytic activity on amide **2**, most had low enantioselectivity. Of these isolates, strains 6-1 and 13-1 were found actively hydrolyze amide **2** *R*-selectively, while strain 23-2 showed high *S*-selective hydrolysis activity toward amide **2**. *E* values $[V_{max(fast)}/K_{m(fast)}]/[V_{max(slow)}/K_{m(slow)}]$, developed by Sih and co-workers [8] as an index for the efficiency of a kinetic resolution, were 83, 4, and 68, respectively. Strains 6-1 and 23-2 were then chosen as candidates R-6 and S-2 for further studies, respectively.

The taxonomical characteristics of strain R-6 were as follows: rod cells (0.7–0.8 × 1.5–2.0 μm), Gram negative, non-spore-forming, and motile. The most variable region of the 16S rDNA sequence revealed 99.9% identity to *Shinella zoogloeoides* IAM 12669 [9]. These characteristics have indicated that strain R-6 belongs to the genus *Shinella* sp. because it is rod-shaped, Gram-negative, non-spore-forming, motile, glucose oxidation-negative, catalase-positive, and oxidase-positive [9]. *Shinella* sp. R-6 was deposited in National Institute of Technology and Evaluation (NITE) under the accession number NITE P-1527.

The taxonomical characteristics of strain S-2 were as follows: rod cells (rod-coccus cycle, 24 h: 0.7–0.8 × 1.5–2.5 μm, 72 h: 0.7–0.8 × 1.0–1.2 μm), Gram positive, non-spore-forming, and non-motile. The most variable region of the 16S rDNA sequence revealed 98.2% identity to *Arthrobacter nitroguajacolicus* G2-1 [10]. These characteristics have indicated that strain S-2 belongs to the genus *Arthrobacter* sp. because it is rod-shaped and rod-coccus cycle, Gram-positive, non-spore-forming, non-motile, glucose oxidation-negative, catalase-positive, and oxidase-negative [10]. *Arthrobacter* sp. S-2 was deposited in National Institute of Technology and Evaluation (NITE) under the accession number NITE P-1526.

3.2. Effect of substrate concentration

The influence of the substrate concentration on hydrolysis activity toward amide **2** was investigated at substrate concentrations between 0.20% and 20% (Table 3). When the reaction in the 5.0 mL scale was carried out using wet cells from 10 mL of the culture, the reaction using *Arthrobacter* sp. S-2 was completed

Table 2
3,3,3-Trifluoro-2-hydroxy-2-methylpropanamide **2** hydrolysis activity from soil samples.

Soil sample	Number of strains			
	Isolated microorganisms	Degradation of **2** (>10%)	Stereoselectivity toward **2** (>50% ee of **1**)	
			R	*S*
50	188	63	2	1
Strain no.	Recovery of 2 (%)	Yield of 1 (%)	Ee of 1 (%)	*E* value
6-1	44.8	41.6	95.4 (*R*)	83
13-1	29.9	33.5	51.2 (*R*)	4
23-2	49.1	47.8	89.8 (*S*)	68

Table 3
Effect of substrate concentration of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide **2** on the hydrolysis activity of *Shinella* sp. R-6 and *Arthrobacter* sp. S-2.

Species	Sub. conc. (%)	Time (h)	Yield of **1** (%)	Ee of **1** (%)
Shinella sp. R-6	0.20	24	54.7	90.6 (*R*)
	0.50	24	47.0	93.3 (*R*)
	1.0	24	33.0	94.6 (*R*)
	2.0	24	18.8	91.1 (*R*)
	5.0	24	7.3	84.0 (*R*)
	10	24	2.6	79.6 (*R*)
Arthrobacter sp. S-2	0.20	4	53.7	90.9 (*S*)
	0.50	4	47.3	95.0 (*S*)
	1.0	8	47.3	96.4 (*S*)
	2.0	24	50.1	96.0 (*S*)
	5.0	24	19.8	96.7 (*S*)
	10	24	3.2	91.8 (*S*)
	20	24	1.2	80.1 (*S*)

smoothly within 24 h using less than 2.0% of the substrate concentration. On the other hand, the reaction using *Shinella* sp. R-6 only proceeded with a substrate concentration of less than 0.5%. Although the high enantioselectivity of the reaction using *Arthrobacter* sp. S-2 was maintained up to a substrate concentration of 10%, that using *Shinella* sp. R-6 decreased to over 2.0%. These results indicated that *Arthrobacter* sp. S-2 can be tolerant and show higher enantioselectivity than *Shinella* sp. R-6 at high substrate concentrations.

3.3. Preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid (*1*)

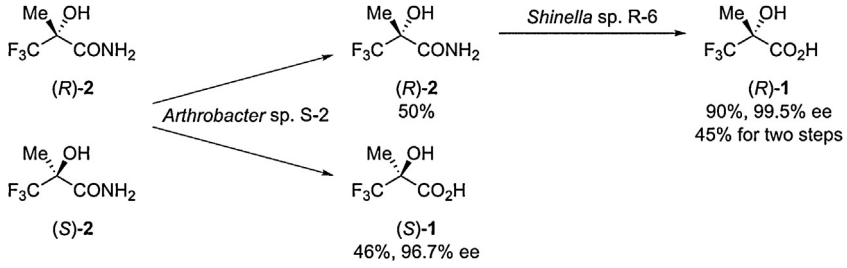
To demonstrate the applicability of the biocatalytic enantioselective hydrolysis of amide **2** to acid **1** catalyzed by *Shinella* sp. R-6 and *Arthrobacter* sp. S-2, we also performed the conversion on a preparative scale using wet cells from an appropriate volume of culture (Scheme 1). Encouraged by the complementary enantioselectivity of the two strains *Shinella* sp. R-6 and *Arthrobacter* sp. S-2, we applied them to the two-step reaction. The first reaction using *Arthrobacter* sp. S-2, selected to increase the productivity, should have produced (*S*)-acid **1** at a substrate concentration of 2.0%. The recovered (*R*)-amide **2** should then have been applied to the reaction using *Shinella* sp. R-6 at a substrate concentration of 0.50%, and converted to (*R*)-acid **1** with increased enantiomeric excess under mild conditions.

The reaction mixture using *Arthrobacter* sp. S-2 in 100 mL consisted of 2.00 g of (±)-amide **2**, 20 mM KPB (pH 7.0), and wet cells from 200 mL of the culture. This was incubated at 30 °C with shaking. Progress was monitored by HPLC analysis. The hydrolysis of (±)-amide **2** had proceeded by approximately 50% after 22 h. After centrifugation, the supernatant was extracted with EtOAc. In this step, only recovered (*R*)-amide **2** could be extracted, and the (*S*)-acid **1** produced remained in the aqueous layer. (*R*)-Amide **2** and (*S*)-acid **1** could be separated easily using only an extraction step. (*S*)-Acid **1** could be extracted from the aqueous layer with Et_2O at pH 4, and its yield and enantiomeric excess were 46% and 96.7%, respectively. The recovered (*R*)-amide **2** was applied to the second reaction using *Shinella* sp. R-6 at a substrate concentration of 0.50%, converted to (*R*)-acid **1** after 34 h, and its yield and enantiomeric excess achieved 45% for two steps and 99.5%, respectively.

4. Discussion

The screening and isolation of two microorganisms that can enantioselectively hydrolyze amide **2** have been described in this study. Although the intramolecular amide bond was generally resistant to hydrolysis and could only be cleaved under harsh conditions, *Shinella* sp. R-6 and *Arthrobacter* sp. S-2, newly isolated from soil samples, exhibited enantioselective hydrolysis activity toward amide **2** under mild conditions. Interestingly, *Shinella* sp. R-6 and *Arthrobacter* sp. S-2 showed opposite enantiopreferences from each other, and could be used in the preparation of both enantiomers of acid **1**. A previously reported amidase from *K. oxytoca* PRS1 showed *R*-selective hydrolysis activity toward amide **2** [5], and the amidase from *Mycobacterium neoaurum* ATCC 25795 also exhibited *R*-selectivity toward 2-amino-3,3,3-trifluoro-2-methylpropanamide containing an amino group not a hydroxyl group [11]. On the other hand, no microorganism or its enzymes exhibiting *S*-selective hydrolysis activity toward amide **2** have been reported to date. *Arthrobacter* sp. S-2 is the first example of a microorganism that exhibits *S*-selective hydrolysis activity toward amide **2**.

The incorporation of fluoride can have marked effects on the properties of fine chemicals and pharmaceuticals. Considering the divergent biological activities of both enantiomers of trifluoromethyl-substituted compounds, the availability of these compounds in their enantiomerically pure form is highly desirable. Although many routes toward enantiomerically pure compounds have been developed using chemical and enzymatic methods, kinetic resolution is still useful for the preparation of both enantiomers. Among them, the enzymatic enantioselective hydrolysis



Scheme 1. The preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid **1** *via* the two-step whole-cell reaction.

of amides has been highly established; however, only a few reports have been investigated the preparation of enantiomerically pure trifluoromethyl-substituted aliphatic acids [4,11]. In addition, it has been reported that the enzymatic hydrolysis of α-hydroxy amide, like lactamide, proceeds at a nearly equal rate toward both enantiomers [12]. We also used a set of incubated microorganisms involving microorganisms such as *Pseudomonas* sp. and *Rhodococcus* sp., which have been used for the hydrolysis of many amides; however, none exhibited high enantioselectivity toward amide **2**, although some showed high hydrolysis activity, as shown in Table 1. Furthermore, although the recovered enantiomer had to be converted to an acid for the preparation of both enantiomers, the reaction proceeded only under harsh conditions such as strong acidic pH and high temperature. Under mild conditions, the two newly isolated microorganisms, *Shinella* sp. R-6 and *Arthrobacter* sp. S-2, could easily prepare both enantiomers of acid **1** as enantiomerically pure forms.

5. Conclusions

This study has described the screening and isolation of microorganisms that enantioselectively hydrolyze amide **2**. The two newly isolated microorganisms, *Shinella* sp. R-6 and *Arthrobacter* sp. S-2 exhibited *R*- and *S*-selective hydrolysis activity toward amide **2**, respectively. The preparation of both enantiomers of acid **1** *via* the two-step whole-cell reaction was investigated using these two strains.

Acknowledgment

This research was partly supported by a Grant-in-Aid for Scientific Research (A) (23248015) from Japan Society for the Promotion of Science (JSPS) to Y. Asano.

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