ORIGINAL ARTICLE

Bioconversion of AS1387392: bioconversion studies involving *Amycolatopsis azurea* JCM 3275

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We screened actinomycetes capable of converting AS1387392 to AS1429716 and identified those strains capable of hydroxylation. *Amycolatopsis azurea* JCM 3275 was found to be a particularly efficient strain, capable of converting AS1387392 to AS1429716, with a yield of 44% after 9 h. This strain can metabolize not only the hydroxylation of phenylalanine at the *meta* and *para* positions but also the reduction of hydroxyketones, as shown by the isolation of bioconversion products. Examination of more suitable conversion conditions showed that pH 7.8 and 25 °C were the optimum pH and temperature for bioconversion, respectively. We also demonstrated the effect of carbon and nitrogen sources in the culture media on hydroxylation. Using this strain, we were able to efficiently produce AS1429716 as a chemical template. Further derivatization studies may provide more effective, safer immunosuppressants than those that are currently on-market. *The Journal of Antibiotics* (2010) 63, 643–647; doi:10.1038/ja.2010.108; published online 6 October 2010

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INTRODUCTION

The novel immunosuppressant AS1387392 (**1**) (Figure 1), isolated from *Acremonium* sp. No. 27082, has been found to exert a strong inhibitory effect against histone deacetylase (HDAC) with a good oral absorption profile.¹ Although AS1429716 (**2**) (Figure 1) presents an attractive chemical template for synthesizing candidate immunosuppressant compounds subsequent to **1**, actually constructing such a compound is difficult from a chemical stance.

Microorganisms are well known to be capable of hydroxylating various compounds.^{2–6} In our screening program, we identified 12 actinomycete strains capable of efficiently converting **1** to **2**.⁷ We then focused our attention on *Amycolatopsis azurea* JCM 3275, a highly efficient strain. Here, we describe the isolation and biological activities of bioconversion products and investigate bioconversion conditions of *A. azurea* JCM 3275.

MATERIALS AND METHODS

Media

Medium A consisted of glucose 0.5%, sucrose 0.5%, oatmeal 0.5%, yeast extract 0.2%, peptone 0.5%, peanut powder 0.5%, humic acid 0.01%, Tween 80 0.1% and CaCO₃ 0.2%, at pH 7.0. Medium B consisted of glycerol 6%, soybean flour 1% and corn steep liquor 3% (Nihon Shokuhin Kako, Tokyo, Japan), at pH 6.5.

Culture and bioconversion conditions

The seed flask containing medium A was inoculated with a loopful of the *A. azurea* JCM 3275 plate culture. After incubation on a shaker at 250 r.p.m. for 3 days at 30 °C, the broth was transferred (2%) into medium B and cultured for a further 2 days at 250 r.p.m. at 30 °C. Both growth stages contained 30 ml of media in a 100-ml Erlenmeyer flask. After these cultivation periods, 14 ml of culture broth and 6 ml of solution containing 170 mM phosphate buffer (pH 6.5) and 1.7 mg ml⁻¹ of compound **1** were transferred to a 100-ml Erlenmeyer flask, and then the reaction mixtures were incubated on a shaker at 250 r.p.m. at 30 °C for 7 h. Bioconversion stage contained 20 ml of reaction mixtures in a 100-ml Erlenmeyer flask. Bioconversion ratio was expressed as the value of the products per initial substrate defined as high-performance liquid chromatography (HPLC) ratio.

Sample analysis

The reaction mixtures (0.5 ml) were combined with an equal volume of acetone and centrifuged (3000 r.p.m., 10 min), after which the supernatant was transferred to HPLC vials. Analysis was performed as previously described.⁷ Under these conditions, compound **1** and **2** were eluted at respective retention times of 9.8 and 4.6 min.

Extraction and isolation of bioconversion products

The conversion reactions were incubated on a shaker for 7 h. After reaction, the 200 ml reaction mixtures from 10 flasks were extracted with an equal volume of acetone and filtered, and the filtrate was concentrated *in vacuo* and applied to a

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Figure 1 Structures of AS1387392 (1) and AS1429716 (2).

column of Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). The column was eluted with both 30 and 40% aqueous acetonitrile.

All eluted fractions containing the bioconversion products were concentrated *in vacuo* to give an aqueous residue. This residue was extracted with ethyl acetate and the extracts were concentrated *in vacuo* to give an oily residue, which was subsequently dissolved in methanol. Compounds 2 to 6 were completely separated using preparative HPLC (column: Mightysil RP-18 GP 250 mm×20 mm I.D. (Kanto Chemical, Tokyo, Japan), flow rate: 10 ml min⁻¹, mobile phase: 25–30% aqueous acetonitrile, detection: Ultraviolet absorption at 210 nm). Each fraction containing bioconversion products was then concentrated *in vacuo* and dissolved in *tert*-butyl alcohol. Subsequent lyophilization produced 2 (34.7 mg), 3 (7.7 mg), 4 (3.5 mg), 5 (16.2 mg) and 6 (4.3 mg) as a white powder.

Compound 3

¹H (500 MHz, CDCl₃) δ 7.57 (1H, d, J=10 Hz, exchangeable), 7.14 (1H, dd, J=8 and 8 Hz), 7.09 (1H, d, J=10 Hz, exchangeable), 6.78 (1H, d, J=8 Hz), 6.71 (1H, s), 6.68 (1H, d, J=8 Hz), 5.88 (1H, s, exchangeable), 5.34 (1H, exchangeable), 5.18 (1H, m), 4.69 (1H, br d, J=8 Hz), 4.24 (1H, m), 4.21 (1H, m), 3.87 (1H, m), 3.57 (1H, exchangeable), 3.32 (1H, m), 3.19 (1H, dd, J=14 and 9 Hz), 2.92 (1H, dd, J=14 and 6 Hz), 2.50 (1H, m), 2.43 (1H, m), 2.35 (2H, m), 2.18 (2H, m), 1.90–1.72 (3H, m), 1.60 (3H, m), 1.39 (3H, d, J=7 Hz), 1.40–1.20 (4H, m), 1.29 (3H, s), and 0.84 (3H, t, J=7.5 Hz); HRESI-MS *m/z* 559.3126 (M+H)⁺, calculated: 558.30535 for C₂₉H₄₂N₄O₇.

Compound 4

¹H (500 MHz, CD₃OD) δ 7.30–7.14 (5H, m), 5.12 (1H, dd, J=8 and 7 Hz), 4.78 (1H, br d, J=8 Hz), 4.32 (1H, t, J=7.5 Hz), 3.68 (1H, m), 3.55 (1H, m), 3.35 (1H, m), 3.32 (1H, m), 3.15 (1H, dd, J=14 and 8 Hz), 2.94 (1H, dd, J=14 and 7 Hz), 2.28 (2H, m), 2.09 (2H, m), 1.84 (1H, m), 1.73 (2H, m), 1.57 (3H, m), 1.44–1.22 (6H, m), 1.26 (3H, s), 1.14 (3H, d, J=6.5 Hz) and 0.79 (3H, t, J=7.5 Hz); HRESI-MS *m/z* 545.3342 (M+H)⁺, calculated 544.32609 for C₂₉H₄₄N₄O₆.

Compound 5

¹H (500 MHz, CDCl₃) δ 7.53 (1H, d, J=10 Hz, exchangeable), 7.10 (2H, d, J=8 Hz), 7.09 (1H, d, J=9 Hz, exchangeable), 6.75 (2H, d, J=8 Hz), 5.85 (1H, s, exchangeable), 5.11 (1H, m), 4.83 (1H, exchangeable), 4.67 (1H, br d, J=8 Hz), 4.20 (1H, m), 3.85 (1H, m), 3.78 (1H, m), 3.60 (1H, m), 3.27 (1H, m), 3.17 (1H, dd, J=14 and 9 Hz), 2.88 (1H, dd, J=14 and 6 Hz), 2.33 (2H, m), 2.17 (2H, m), 1.93–1.72 (5H, m), 1.65–1.50 (3H, m), 1.43–1.27 (6H, m), 1.28 (3H, s), 1.15 (3H, d, J=6.5 Hz) and 0.83 (3H, t, J=7.5 Hz); HRESI-MS *m/z* 561.3286 (M+H)⁺, calculated 560.32100 for C₂₉H₄₄N₄O₇.

Compound 6

¹H (500 MHz, CDCl₃) δ 7.56 (1H, d, J=10 Hz, exchangeable), 7.15 (1H, dd, J=8 and 8 Hz), 7.08 (1H, d, J=10 Hz, exchangeable), 6.80 (1H, d, J=8 Hz), 6.71 (1H, s), 6.69 (1H, d, J=8 Hz), 5.88 (1H, s, exchangeable), 5.20 (1H, exchangeable), 5.18 (1H, m), 4.68 (1H, br d, J=8 Hz), 4.21 (1H, m), 3.85 (1H, m), 3.60 (1H, m), 3.32 (1H, m), 3.30 (1H, m), 3.17 (1H, dd, J=14 and 9 Hz), 2.93 (1H, dd, J=14 and 6 Hz), 2.32 (2H, m), 2.22–2.10 (4H, m), 1.88–1.73 (3H, m), 1.65–1.45 (3H, m), 1.44–1.27 (6H, m), 1.28 (3H, s), 1.20 (3H, d, J=6.5 Hz) and 0.83 (3H, t, J=7.5 Hz); HRESI-MS *m/z* 561.3286 (M+H)⁺, calculated 560.32100 for C₂₉H₄₄N₄O₇.

Assay for HDAC and splenocyte proliferation

AS1387392 exerted potent inhibitory activity against HDAC and splenocyte proliferation stimulated by anti-CD3 antibody, which induced T-cell activation. Given this observed activity, the *in vitro* immunosuppressive activities of bioconversion products were examined by assaying HDAC and splenocyte proliferation by the method previously proposed by Mori *et al.*⁸ Briefly, HDAC and [³H]-acetyl histones were prepared from human T-cell leukemia Jurkat cells. HDAC activity was determined on the basis of the release of [³H]-acetic acid. Immunosuppressive activity was examined by assessing anti-CD3 antibody-stimulated splenocyte proliferation, induced when the antibody binds T-cell receptors and activates the proliferation response. Splenocytes harvested from female BALB/c mice (Charles River Japan, Kanagawa, Japan) were cultured in Roswell Park Memorial Institute (RPMI)-1640 media containing 1 μg ml⁻¹ anti-CD3 antibody at 37 ° for 3 days in a CO₂ incubator. After being cultured, proliferation was measured using alamar blue (Promega, Fitchburg, WI, USA).

RESULTS

Time-course of bioconversion products

Compound 2 was a major conversion product derived from *A. azurea* JCM 3275. After 9 h, compound 1 had disappeared entirely and compound 2 had achieved its peak bioconversion ratio (44%) after reaction initiation. HPLC analysis also revealed other bioconversion products (3–6), the time courses of which are shown in Figure 2. Levels of compound 4 increased rapidly and peaked at 3 h, disappearing after 9 h. Compound 3 achieved its peak conversion ratio at 9 h, similar to 2, with the ratio gradually decreasing thereafter. In contrast, conversion ratios of compounds 5 and 6 increased as the reaction progressed.

Isolation of bioconversion products

The reaction mixtures were extracted with acetone and filtered, and concentrated extract was fractionated using a Diaion HP-20 (Mitsubishi Chemical) with 30 and 40% acetonitrile. The eluted fractions were concentrated *in vacuo* to give an aqueous residue and extracted with ethyl acetate. Compounds 2 to 6 were completely separated using preparative HPLC, yielding the bioconversion products 2 (34.7 mg), 3 (7.7 mg), 4 (3.5 mg), 5 (16.2 mg) and 6 (4.3 mg) as a white powder.

The physicochemical properties of the bioconversion products are described in Table 1. Ultraviolet absorption values were the same for compounds 1 and 4, whereas values for all other bioconversion products were approximately 280 nm, a discriminative wavelength. The physicochemical properties of 2 were reported previously.⁷

HRESI-MS data revealed the molecular formula of 3 to be C₂₉H₄₂N₄O₇, the same as that of 2. The ¹H nuclear magnetic resonance spectrum of 3 was quite similar to that of 2 except for the benzene moiety. The position of hydroxylation was determined to be *meta* on the basis of the three contiguous aromatic protons (6.68, 7.14 and 6.78 p.p.m.) and one isolated (6.71 p.p.m.). The presence of a *meta*-tyrosine was further corroborated by long-range couplings of benzylic

methylene protons (3.19 and 2.92 p.p.m.) to a singlet benzene proton (6.71 p.p.m.) and a doublet one (6.78 p.p.m.) in the ^1H - ^1H COSY (^1H nuclear magnetic resonance data; see the Materials and methods section).

HRESI-MS measurement of **4** afforded a molecular formula $\text{C}_{29}\text{H}_{44}\text{N}_4\text{O}_6$ indicating the presence of two extra hydrogen atoms compared with **1**. Analysis of ^1H - ^1H COSY data indicated the presence of $\text{CH}_3\text{-CH(OH)-CH(OH)-}$ (1.14-3.55-3.35 p.p.m.), indicating that Aoh (2-amino-8-oxo-9-hydroxydecanoic acid) in **1** was transformed into Add (2-amino-8,9-dihydroxydecanoic acid).

On the basis of ^1H nuclear magnetic resonance data analysis, transformation pattern of **5** was a combination of **2** and **4**. The unit was oxidized to Tyr (δ 7.10 (2H, d, $J=8$ Hz) and 6.75 (2H, d, $J=8$ Hz)) and Aoh unit was changed to Add.

HRESI-MS analysis of **6** afforded a molecular formula $\text{C}_{29}\text{H}_{44}\text{N}_4\text{O}_7$, the same as that of **5**. Analysis of ^1H - ^1H COSY data indicated the presence of a *meta*-tyrosine and Add (^1H nuclear magnetic resonance data, see the Materials and methods section).

Figure 2 Time course of bioconversion products catalyzed by *Amycolatopsis azurea* JCM 3275. The bioconversion activity catalyzed by *A. azurea* JCM 3275 was assessed by high-performance liquid chromatography method. The Y-axis on the left indicates the bioconversion ratios of compound **2-6**, expressed as the value of the products per initial substrate. The Y-axis on the right indicates the ratio of the residual substrate (compound **1**).

Figure 3 Effects of reaction pH (a) and temperature (b) on activity of the hydroxylation catalyzed by *A. azurea* JCM 3275 (dashed line indicates control activity at 30 °C and pH 6.5). The hydroxylation activity was estimated as the value of compound **2** per initial substrate in various conditions.

Table 1 Physicochemical properties of compounds **2-6**

	Value				
	Compound 2 ^a	Compound 3	Compound 4	Compound 5	Compound 6
Appearance	white powder	white powder	white powder	white powder	white powder
HR-ESI-MS (<i>m/z</i>)					
Found	559.3122 [M+H] ⁺	559.3126 [M+H] ⁺	545.3342 [M+H] ⁺	561.3286 [M+H] ⁺	561.3286 [M+H] ⁺
Calculated	559.3132 [M+H] ⁺	559.3126 [M+H] ⁺	545.3334 [M+H] ⁺	561.3283 [M+H] ⁺	561.3283 [M+H] ⁺
Molecular formula	$\text{C}_{29}\text{H}_{42}\text{N}_4\text{O}_7$	$\text{C}_{29}\text{H}_{42}\text{N}_4\text{O}_7$	$\text{C}_{29}\text{H}_{44}\text{N}_4\text{O}_6$	$\text{C}_{29}\text{H}_{44}\text{N}_4\text{O}_7$	$\text{C}_{29}\text{H}_{44}\text{N}_4\text{O}_7$
UV λ_{max} (MeOH) nm (log ϵ)	223 (sh), 276 (3.75)	276 (3.15)	237 (sh)	224 (sh), 279 (3.40)	275 (3.26)
HPLC retention time (min) ^b	2.8	3.2	4.1	2.4	2.5

Abbreviations: HPLC, high-performance liquid chromatography; HR-ESI-MS, high-resolution electrospray ionization mass spectrometry; UV, ultraviolet.

^aFrom Sasamura et al.⁷

^bHPLC condition: performed as the Materials and methods section except for the use of mightysil RP-18 GP 150 mm×4.6 mm as column and 40% aqueous acetonitrile as mobile phase.

Effect of pH and temperature on bioconversion

Reaction conditions, such as pH and temperature, generally affect the rate of bioconversion. Investigation into reaction conditions most suitable for bioconversion showed that activity for converting **1** to **2** was highest at pH 7.8 (Figure 3a). This activity was observed in a temperature range of approximately 15–35 °C and proceeded most efficiently at 25 °C (Figure 3b).

Figure 4 Effects of various carbon sources in culture media on hydroxylation activity catalyzed by *A. azurea* JCM 3275 (dashed line indicates control activity using 6% glycerol as carbon source). The hydroxylation activity was estimated as the value of compound **2** per initial substrate.

Figure 5 Effects of various nitrogen sources in culture media on hydroxylation activity catalyzed by *A. azurea* JCM 3275 (dashed line indicates control activity using 3% corn steep liquor (CSL) and 1% soybean meal as nitrogen source). The hydroxylation activity was estimated as the value of compound **2** per initial substrate.

Effect of carbon and nitrogen sources in the culture media on hydroxylation

To assess the optimum culture media for hydroxylation, the bioconversion activity was examined while culturing *A. azurea* JCM 3275 in media containing various carbon and nitrogen sources. Hydroxylation activity using media with Pindex#3 was as efficient as that in control media (glycerol 6%), whereas activity with other carbon sources was even lower (Figure 4). When corn steep liquor and soybean flour in medium B were exchanged for other nitrogen sources (2.5%)—namely soybean flour, pharmamedia and dried yeast—hydroxylation activity increased in efficiency. The culture in media using peanut powder gave the highest activity (Figure 5).

Immunosuppressive activity of bioconversion products

Compound **2** inhibited the activity of human partially purified HDAC ($IC_{50}=12 \text{ ng ml}^{-1}$) and potently inhibited splenocyte proliferation ($IC_{50}=1.2 \text{ ng ml}^{-1}$), demonstrating this compound to be as potent as **1** ($IC_{50}=1 \text{ ng ml}^{-1}$ in splenocyte proliferation and $IC_{50}=18 \text{ ng ml}^{-1}$ in HDAC assay). Compound **3** also inhibited splenocyte proliferation ($IC_{50}=3.6 \text{ ng ml}^{-1}$). No obvious inhibitory effects were observed for compounds **4**, **5** or **6** at 1000 ng ml^{-1} .

DISCUSSION

Here, we described our bioconversion study examining *A. azurea* JCM 3275. This strain was found to be able to convert **1** to **2** (44% yield at 9 h) and produce other bioconversion compounds (**3–6**) at the same time. We also drafted potential structures and metabolic pathways of the isolated compounds (Figure 6). These results suggest that *A. azurea* JCM 3275 is able to metabolize three positions on **1**: reduction of a hydroxyketone and hydroxylation of phenylalanine at the *para* and *meta* positions.

In our investigation of conditions more suitable for bioconversion than those known at present, the optimum pH and temperature for conversion were found to be pH 7.8 and 25 °C, respectively. In addition, we determined optimum carbon and nitrogen sources in culture media for hydroxylation. These results will facilitate more effective production of **2**.

With regard to the time course, compound **2** achieved its maximum conversion ratio after 9 h, with the ratio decreasing thereafter mainly because of the reduction of hydroxyketone (conversion to **5**). Further improvement in conversion efficiency when using *A. azurea* JCM 3275 will require regulation of other bioconversion products. In particular, suppression of hydroxyketone reduction may improve the conversion yield, and the cloning and heterologous expression of the hydroxylation enzyme may help improve production of **2**.⁹

The immunosuppressive activities of both **2** and **3** were as potent as **1**. However, no activity was noted for **4**, **5** or **6**, which were formed following reduction of a hydroxyketone, indicating that a hydroxyketone is essential for HDAC inhibition. Results of other studies have suggested that the epoxyketone group in trapoxin is necessary for HDAC inhibition.¹⁰ Our results, here, indicate that the hydroxyketone group may have the same role as epoxyketone.

In the present study, we were able to effectively produce AS1429716 as a chemical template by using the *A. azurea* strain JCM 3275. We obtained promising results in our study of natural product derivatization using bioconversion methods, suggesting that microbial transformations can indeed complement chemical modifications. Further derivatization studies may provide more effective and safer immunosuppressants than those that are currently available on-market.

Figure 6 Proposed structures and metabolic pathways of bioconversion products.

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