

## ORIGINAL ARTICLE

# Bioconversion of AS1387392: screening and characterization of actinomycetes that convert AS1387392 to AS1429716

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AS1387392 was a novel and powerful histone deacetylase inhibitor with an excellent oral absorption profile, but this compound was lacking in active moieties, which are essential to synthesize more derivatives. In our screening program to identify actinomycetes capable of converting AS1387392 to AS1429716, which has an active moiety to synthesize more derivatives, we identified 12 strains capable of efficient hydroxylation. Results of phylogenetic analysis of 16S rDNA sequences suggested that these strains belonged to the genera *Lentzea*, *Saccharopolyspora*, *Sphaerisporangium* and *Amycolatopsis*. Morphological and chemical characteristics as well as results of phylogenetic analysis suggested that strain No. 7980 was a new species belonging to the genus *Amycolatopsis*, according to the FASTA search result of 16S rDNA gene sequence. Using these strains, we can easily produce AS1429716 as a chemical template for further chemical modifications, which may provide more effective and safer immunosuppressant.

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**Keywords:** *Acremonium*; AS1387392; AS1429716; bioconversion; histone deacetylase inhibitor; immunosuppressant

## INTRODUCTION

The immunosuppressants tacrolimus and cyclosporin A dramatically changed the field of organ transplants. However, problems such as chronic rejection and other side effects based on the mode of action of these calcineurin inhibitors remain unresolved.<sup>1,2</sup> To search for more effective, safer therapeutic agents, we continue to explore other novel immunosuppressants.

Through our efforts to address these problems, we previously identified the novel compound AS1387392, (Figure 1) a cyclic tetrapeptide produced by the fungi *Acremonium* sp. No. 27082.<sup>3</sup> This compound exhibited a strong inhibitory effect against histone deacetylase and splenocyte proliferation. Further, AS1387392 showed good oral absorption, with a plasma concentration higher than that of FR235222, (Figure 1) an analog of AS1387392.<sup>4–6</sup>

As it turned out that AS1387392 would be a good chemical template, the derivatization of this compound was started. AS1387392, however, is lacking in active moieties, which are essential to synthesize more derivatives. After some feasibility studies, AS1429716 (Figure 1) with a good chemical moiety was synthesized and the activity of this compound showed as potent as that of AS1387392 in immunosuppressive activity using splenocyte

proliferation assay. Although AS1429716 was obtained as an attractive template, the yield of the product by chemical synthesis method was very low. To overcome this problem, we started to study the bioconversion method to improve it.

Microorganisms are well known to be capable of hydroxylating a range of compounds.<sup>7–11</sup> Bioconversion is practical for position- and stereo-selective hydroxylation and is a powerful tool for contributing to chemical template and metabolite production. We therefore screened actinomycetes capable of converting AS1387392 to AS1429716.

Here, we describe the screening and phylogenetic analysis for actinomycete strains, which convert AS1387392 to AS1429716, the taxonomy of strain No. 7980, and the isolation and physicochemical properties of AS1429716 in this paper.

## MATERIALS AND METHODS

### Microorganisms used for bioconversion

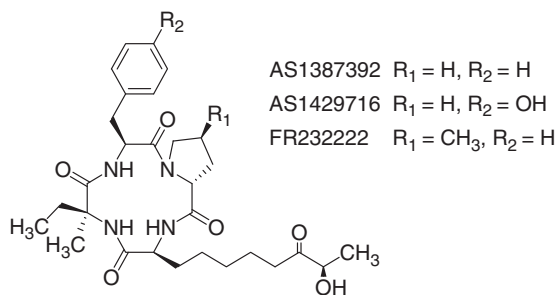
In this study, we examined ~8000 actinomycete strains isolated from soil in Japan and about 300 type strains purchased from a recognized culture collection such as American Type Culture Collection (ATCC) and Japan Collection of Microorganisms (JCM).

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**Figure 1** Structures of AS1387392, AS1429716 and FR235222.

### Culture media

Medium A consisted of commmeal 1%, MS#3600 6% (Nihon Shokuhin Kako, Tokyo, Japan), Pharmamedia 1.2% (Traders Protein, Lubbock, USA), dried yeast 0.8%, KH<sub>2</sub>PO<sub>4</sub> 0.3%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.3% and FeSO<sub>4</sub>·7H<sub>2</sub>O 0.02%, pH 6.5. Medium B consisted of glucose 2%, MS#3600 5%, potato protein 1.5%, wheat germ 1.5% and CaCO<sub>3</sub> 0.2%, pH 6.5. Medium C consisted of glucose 0.5%, MS#3600 2.5%, dried yeast 0.5%, soybean flour 1%, L-asparagine 0.2%, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.0005%, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.001%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05% and CaCO<sub>3</sub> 0.2%, pH 6.5. Medium D 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 CaCO<sub>3</sub> 0.2%, pH 7.0. Medium E consisted of glycerol 6%, soybean flour 1% and corn steep liquor 3% (Nihon Shokuhin Kako, Tokyo, Japan), pH 6.5.

### Culture and bioconversion conditions for screening

Actinomycete strains were grown in tubes (17 mm×100 mm) containing 1.5 ml of media A, B or C. The culture tubes were inoculated with a loopful of the actinomycete plate culture, and then incubated on a shaker at 250 r.p.m. at 30 °C for 4 or 6 days, depending on cell growth. After incubation, 0.35 ml of culture broth was transferred to another tube containing 170 mM phosphate buffer (pH 6.5) and 1.7 mg ml<sup>-1</sup> AS1387392 in 0.15 ml of deionized water.

### Sample analysis for screening

The reaction mixtures were combined with 0.5 ml of acetone and centrifuged (3000 r.p.m., 10 min), and then the supernatant was transferred to HPLC vials. Primary analysis was performed using our HPLC system (L-4000 UV detector, L-6000 pump and L-7300 column oven; Hitachi High-Technologies, Tokyo, Japan) using an ODS column (Mightysil RP-18 GP 250 mm×4.6 mm ID; Kanto chemical, Tokyo, Japan). The mobile phase was 45% aqueous acetonitrile and the flow rate was 1.0 ml min<sup>-1</sup>. The detection wavelength was set at 210 nm and the column was heated at 50 °C. Under these conditions, AS1429716 and AS1387392 were eluted at a retention time of 4.6 and 9.8 min. Secondary analysis was performed by liquid chromatography-mass spectrometry (YMC ODS-AM AM-3C1-3 100 mm×2.0 mm ID, 3 μm (YMC, Kyoto, Japan; flow rate: 0.2 ml min<sup>-1</sup>, mobile phase: 30% aqueous acetonitrile, detection: MS and UV absorption at 210 nm, column temperature: 50 °C) using an Agilent HP1100 (Hewlett-Packard, Palo Alto, CA, USA). Under these conditions, AS1429716 was eluted at a retention time of 8.1 min and showed a molecular ion peak at *m/z* 559 [M+H]<sup>+</sup>.

### 16S rDNA sequencing and phylogenetic analysis

The 16S rDNA sequence was determined using the method developed by Nakagawa *et al.*<sup>12</sup> 16S rDNA sequences of type strains were obtained from the DNA Data Bank of Japan database, (<http://www.ddbj.nig.ac.jp>) and a phylogenetic tree was constructed using the alignment program ClustalW.<sup>13</sup>

### Taxonomic characteristics of strain No. 7980

Strain No. 7980 was isolated from a soil sample collected in Nagano Prefecture, Japan. We conducted our taxonomic study of strain No. 7980 using the methods and media described by Shirling and Gottlieb,<sup>14</sup> and Waksman.<sup>15</sup>

All observations were made after 14 days' cultivation at 30 °C. Morphological observations were made using an optical microscope and a scanning electron microscope on cultures grown on yeast extract–starch agar containing 0.08 g of yeast extract (Daigo Eiyo, Osaka, Japan), 0.4 g of soluble starch and 16 g of agar in 1 l of tap water (pH adjusted to 7.2 with 1N NaOH before sterilization). The range of temperature for growth was determined on yeast extract–starch agar containing 2 g of yeast extract, 10 g of soluble starch and 16 g of agar in 1 l of tap water (pH adjusted to 7.2 with 1N NaOH before sterilization). Carbon utilization was determined on Pridham and Gottlieb's medium.<sup>16</sup> All color names used in this study were taken from *Methuen Handbook of Colour*.<sup>17</sup> Cell preparation and diaminopimelic acid isomer detection were conducted following the procedure developed by Becker *et al.*<sup>18</sup>

### Culture and bioconversion using strain No. 7980

Aqueous seed medium D (30 ml) was poured into a 100-ml Erlenmeyer flasks and sterilized at 121 °C for 30 min. The seed flasks were then inoculated with a loopful of the strain No. 7980 plate culture and shaken on a shaker at 30 °C for three days. After incubation, 0.6 ml of the seed culture was then transferred to 100-ml Erlenmeyer flasks containing 30 ml of medium E, and these flasks were shaken at 30 °C for two days. After this cultivation, 35 ml of the culture broth and 15 ml of solution containing 170 mM phosphate buffer (pH 6.5) and 1.3 mg ml<sup>-1</sup> AS1387392 were transferred to 500-ml baffled flasks, and then incubated at 30 °C for 5 h. Bioconversion stage contained 50 ml of reaction mixtures in a 500-ml baffled flask.

## RESULTS

### Screening of microorganisms for ability to convert AS1387392 to AS1429716

Preliminary screening was carried out for ~300 strains of fungi and actinomycetes isolated from soil and leaf. Of these, only five strains of actinomycetes were able to convert AS1387392 to AS1429716. Given these findings, we decided to conduct subsequent screening using only actinomycete strains.

To assess bioconversion activity, we conducted HPLC analysis on ~8000 strains isolated from soil in Japan and about 300 type strains of various actinomycete species. After secondary analysis using liquid chromatography–mass spectrometry, we analyzed phylogenetically 12 strains, including 3 type strains, capable of converting AS1387392 to AS1429716.

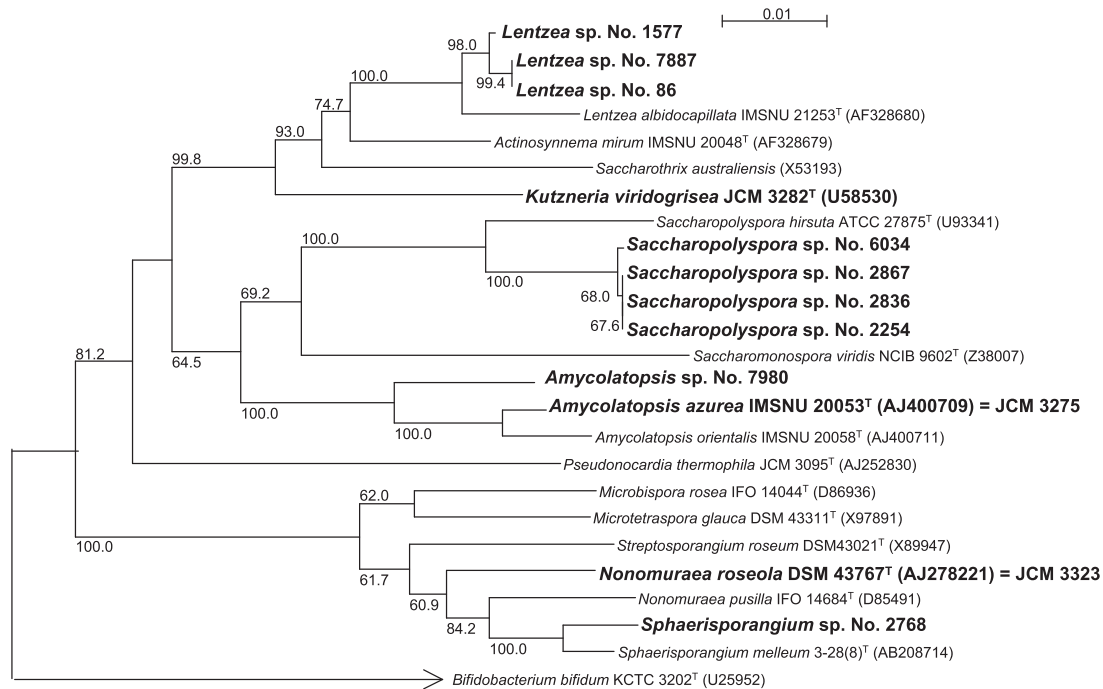
### Phylogenetic analysis of bioconversion strains

Phylogenetic analysis based on 16S rDNA sequences showed that the strains capable of bioconversion belonged to the genera *Lentzea*, *Saccharopolyspora*, *Sphaerisporangium* and *Amycolatopsis*. A phylogenetic tree is shown in Figure 2, and a complete list of the strains capable of bioconversion is shown in Table 1. Relevant strains included high converting yield strains, such as *Lentzea* sp. No. 86, *Sphaerisporangium* sp. No. 2768, *Amycolatopsis azurea* JCM 3275 and *Amycolatopsis* sp. No. 7980. No *Streptomyces* were found capable of converting AS1387392 to AS1429716.

### Taxonomic characteristics of strain No. 7980

The substrate mycelium developed well and branched irregularly, and hyphal segment formation was observed. This strain produced few aerial mycelia, and no spores were observed. Sclerotic granules, sporangia and motile spores or fragments were also not observed.

Cultural and physiological characteristics are described in Tables 2 and 3, respectively. Aerial mycelia were rarely observed. Reverse side growth color ranged from yellowish-white to orange. No melanoid pigments were produced in the tryptone-yeast extract broth or peptone-yeast extract–iron agar (ISP-6). Soluble pigments were produced in the inorganic salts–starch agar (ISP-4). Mycelial mass color



**Figure 2** Phylogenetic tree showing actinomycete strains capable of converting AS1387392 to AS1429716 based on 16S rDNA sequence (almost full-length ca 1400 nucleotides). The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (above 50% are indicated). Bar, 0.01 substitutions per nucleotide position.

**Table 1** Actinomycetes strains capable of converting AS1387392 to AS1429716

Microorganism	Bioconversion ratio <sup>a</sup>
<i>Lentzea</i> sp. No. 86	++
<i>Lentzea</i> sp. No. 1577	+
<i>Lentzea</i> sp. No. 7887	+
<i>Amycolatopsis azurea</i> JCM 3275	++
<i>Amycolatopsis</i> sp. No. 7980	++
<i>Saccharopolyspora</i> sp. No. 2254	+
<i>Saccharopolyspora</i> sp. No. 2836	+
<i>Saccharopolyspora</i> sp. No. 2867	+
<i>Saccharopolyspora</i> sp. No. 6034	+
<i>Kutzneria viridogrisea</i> JCM 3282	+
<i>Nonomuraea roseola</i> JCM 3323	+
<i>Sphaerisporangium</i> sp. No. 2768	++

<sup>a</sup>+, 0.5–15%; ++, > 15%. Bioconversion ratio was expressed as the value of the AS1429716 per initial AS1387392.

**Table 2** Cultural characteristics of strain No. 7980

Medium	Growth	Aerial mycelia	Color of reverse surface	Soluble pigment
ISP-2	Weak	Not observed	Orange (5B8)	Not detected
ISP-3	Weak	Not observed	Yellowish-white (3A2)	Not detected
ISP-4	Weak	Not observed	Yellowish-white (4A2)	Brown (6D7)
ISP-5	Weak	Not observed	Light orange (5A4)	Not detected
ISP-6	Weak	Not observed	Orange (5A6)	Not detected
ISP-7	Weak	Not observed	Light yellow (4A5)	Not detected

Abbreviations: ISP-2, yeast–malt extract agar; ISP-3, oatmeal agar; ISP-4, inorganic salts–starch agar; ISP-5, glycerol–asparagine agar; ISP-6, peptone–yeast extract–iron agar; ISP-7, tyrosine agar

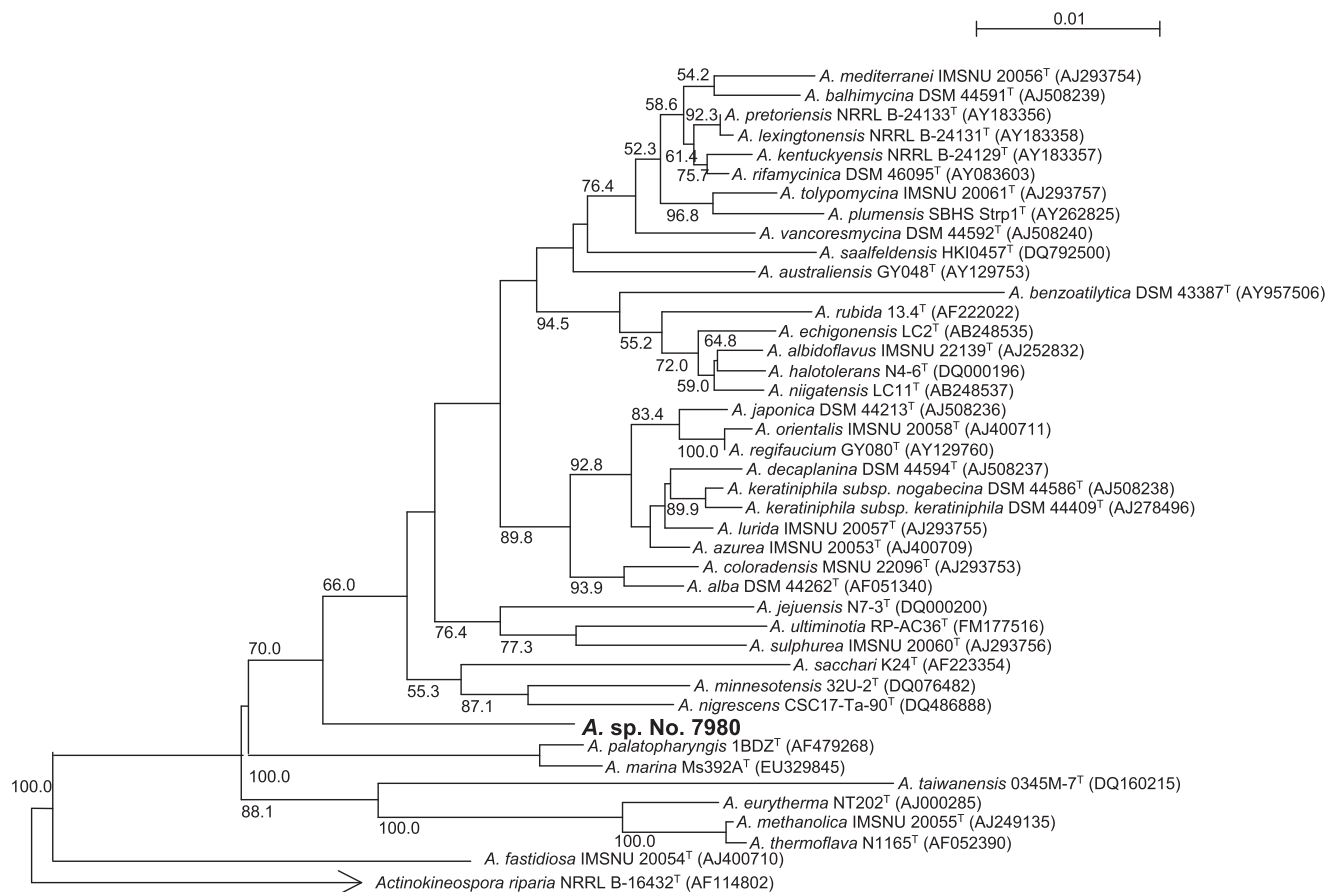
**Table 3** Physiological characteristics of strain No. 7980

Characteristic	Value
Temperature range for growth (°C)	8–34
Optimum temperature for growth (°C)	32
Melanoid pigments produced	–
Soluble pigments produced	+
<b>Carbon utilization</b>	
<i>D</i> -glucose	+
Sucrose	–
<i>D</i> -xylose	±
<i>D</i> -fructose	+
<i>L</i> -rhamnose	–
Raffinose	–
<i>L</i> -arabinose	+
Inositol	+
<i>D</i> -mannitol	+

+, utilized; –, not utilized; ±, weakly utilized

was not pH sensitive. Meso-diaminopimelic acid was detected in whole-cell hydrolysates of this strain. Similarity values of 16S rDNA between strain No. 7980 and members of the genus *Amycolatopsis* were 93.2–96.1%, and both formed a single cluster on the phylogenetic tree (Figure 3).

On the basis of the morphological and chemical characteristics and phylogenetic analysis described above, we concluded that strain No. 7980 belonged to the genus *Amycolatopsis*.<sup>19,20</sup> Therefore, this strain was designated as *Amycolatopsis* sp. No. 7980.



**Figure 3** Phylogenetic tree showing the position of strain No. 7980 based on 16S rDNA sequence (almost full-length *ca* 1400 nucleotides). The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (above 50% are indicated). Bar, 0.01 substitutions per nucleotide position.

### Isolation and physicochemical properties of AS1429716

After reaction, the 200 ml mixtures obtained from four baffled flasks were extracted with 200 ml of methanol. The methanol extract filtrate was concentrated to an aqueous solution and extracted with ethyl acetate. The organic layer was concentrated and applied to a column of 20 ml Daiso ODS-B (Daiso, Osaka, Japan). The column was eluted with 30% aqueous acetonitrile. Further purification was accomplished by preparative HPLC (Mightysil RP-18 GP 250 mm × 4.6 mm ID; Kanto Chemical; flow rate: 1 ml min<sup>-1</sup>, mobile phase: 25% aqueous acetonitrile, temperature: 50 °C, detection: UV absorption at 210 nm). The active fraction was concentrated *in vacuo* and extracted using ethyl acetate, and the solvent layer was dried up and dissolved in *tert*-butyl alcohol. Subsequent lyophilization produced 4.7 mg of AS1429716 as a white powder.

Physicochemical properties of AS1429716 are described in Table 4. The compound was soluble in methanol, ethyl acetate and dimethyl sulfoxide, and slightly soluble in water. Positive-color reactions were observed with iodine vapor, FeCl<sub>3</sub> and Ce(SO<sub>4</sub>)<sub>2</sub>-H<sub>2</sub>SO<sub>4</sub>, and negative reactions during Molisch's, Ehrlich's and Ninhydrin reactions. The observed specific rotation was -75° (*c* 0.2, methanol), and the exact molecular mass (as determined by ESI-TOF-MS) was 559.3122 Da ([M+H]<sup>+</sup>), corresponding to the molecular formula C<sub>29</sub>H<sub>42</sub>N<sub>4</sub>O<sub>7</sub> (theoretical [M+H]<sup>+</sup> = 559.3132 Da).

**Table 4** Physicochemical properties of AS1429716

Characteristic	Value
Appearance	White powder
[α] <sub>D</sub> <sup>23</sup>	-75° ( <i>c</i> 0.2, MeOH)
ESI-TOF-MS ( <i>m/z</i> )	
Observed	559.3122 [M+H] <sup>+</sup>
Calculated	559.3132 [M+H] <sup>+</sup>
Molecular formula	C <sub>29</sub> H <sub>42</sub> N <sub>4</sub> O <sub>7</sub>
UV λ <sub>max</sub> (MeOH) nm (log ε)	223 (sh), 276 (3.75)
Color test	
Positive	I <sub>2</sub> , Ce(SO <sub>4</sub> ) <sub>2</sub> -H <sub>2</sub> SO <sub>4</sub> , FeCl <sub>3</sub>
Negative	Molisch's reaction, Ninhydrin reaction, Ehrlich's reaction
Solubility	
Soluble	Methanol, ethyl acetate, dimethyl sulfoxide
Slightly soluble	Water
TLC (Rf value) <sup>a</sup>	0.23 <sup>b</sup>

<sup>a</sup>Plate: silica gel 60 F<sub>254</sub> (E. Merck, Darmstadt, Germany), ethyl acetate

<sup>b</sup>Rf value of AS1387392 was 0.42 from reference 3.

**Table 5**  $^1\text{H}$ ,  $^{13}\text{C}$  data of AS1429716 and  $^{13}\text{C}$  data of AS1387392

Position	AS1429716		AS1387392
	$\delta_{\text{H}}^{\text{a}}$	$\delta_{\text{C}}^{\text{b}}$	$\delta_{\text{C}}^{\text{c}}$
Aoh	1		174.1
	2	4.20	54.4
	3	1.83, 1.60	28.8
	4	1.30	25.3
	5	1.33	28.8
	6	1.62	23.2
	7	2.50, 2.44	37.3
	8		212.4
	9	4.23	72.6
	10	1.39	19.8
	11	7.10	
Pro	12		171.8
	13	4.68	57.8
	14	2.35, 1.76	24.7
	15	2.20, 1.80	25.0
	16	3.83, 3.27	46.9
	17		
	18	—	—
	Tyr	19	
20		5.12	53.6
21		3.17, 2.88	34.9
22			129.0
23, 27		7.08	130.3
24, 26		6.73	115.4
25			154.4
28		7.54	
Iva		29	
	30		63.1
	31	2.31, 2.17	27.9
	32	0.85	8.4
	33	1.29	22.4
	34	5.88	

<sup>a</sup> $\delta_{\text{H}}$  500 MHz in  $\text{CDCl}_3$ .<sup>b</sup> $\delta_{\text{C}}$  125 MHz in  $\text{CDCl}_3$ .<sup>c</sup> $\delta_{\text{C}}$  from reference 3. $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded with a Bruker AVANCE 500 NMR spectrometer with tetramethylsilane used as an internal standard.

### Structure elucidation

ESI-TOF-MS analysis of AS1429716 afforded a molecular formula  $\text{C}_{29}\text{H}_{42}\text{N}_4\text{O}_7$  indicating the presence of an extra oxygen atom compared with AS1387392 as previously reported.<sup>3</sup> Judging from the  $^1\text{H}$  NMR spectra, the position of the oxygen was determined to be para-position of benzene moiety. An A2B2  $^1\text{H}$  signal pattern ( $\delta$  7.08 (2H, d,  $J=8$  Hz) and 6.73 (2H, d,  $J=8$  Hz)) was typical of 1, 4-disubstituted benzene unit. Replacement of phenylalanine in AS1387392 by tyrosine gave the structure of AS1429716. The structure was further supported by the fact that  $^{13}\text{C}$  data of AS1429716 were quite similar to that of AS1387392 except for tyrosine part. Complete  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignment was made by COSY, HSQC, HMBC and summarized in Table 5 together with  $^{13}\text{C}$  data of AS1387392

### DISCUSSION

In the field of immunosuppressant, AS1429716 is needed as chemical template; however, this compound is difficult to achieve by chemical

reaction. Given that previous studies have found bioconversion to be extremely successful in hydroxylation of various compounds, we considered that production of AS1429716 from AS1387392 with bioconversion to help further chemical analyses. To identify microorganisms capable of converting AS1387392 to AS1429716, we screened some 8000 actinomycete strains. Of these, 12 were found capable of hydroxylation, and the activity of 7 was 'highly efficient'.

Phylogenetic analysis based on 16S rDNA sequence was performed on the strains found capable of this conversion. Interestingly, all of these strains were non-*Streptomyces* actinomycetes, with no *Streptomyces* identified during screening though *Streptomyces* have been found to be able to hydroxylate various compounds.<sup>21–23</sup> More than half of 300 actinomycete type strains we have screened, belong to the genus *Streptomyces*. On application of conventional isolation techniques, more than half of the strains from soil belonged to the genus *Streptomyces*.<sup>24</sup> These previous findings therefore indicated that non-*Streptomyces* strains were ideal for screening for ability to convert AS1387392 to AS1429716, at least under the present culture and bioconversion conditions.

Observations of morphological and chemical characteristics and phylogenetic analysis indicated that strain No. 7980 belonged to the genus *Amycolatopsis*.<sup>19,20</sup> The criterion for similarity using comparison of 16S rDNA sequence is at least 97% homology may indicate that two species are different in the present species definition in bacteriology.<sup>25</sup> No *Amycolatopsis* species showed more than 97% similarity value with that of strain No. 7980. We therefore postulated that strain No. 7980 represented a new species under the genus *Amycolatopsis*.

In this study, we successfully found bacterial strains capable of producing AS1429716, which until now had been chemically inaccessible. With these new findings, further derivation studies may yield still more effective and safer immunosuppressant.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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