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Structure of YM-254890, a Novel $G_{q/11}$ Inhibitor from *Chromobacterium* sp. QS3666

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Abstract—The isolation and structure elucidation of YM-254890, a novel $G_{q/11}$ inhibitor from *Chromobacterium* sp. QS3666, is described. The gross structure was determined by one- and two-dimensional NMR studies and mass spectrometry. YM-254890 is a cyclic depsipeptide containing uncommon amino acids; β-hydroxyleucine (two residues), *N*,*O*-dimethylthreonine and *N*-methyldehydroalanine. YM-254890 exists as a mixture of two conformers in a variety of NMR solvents, and the distinction between major and minor conformers appears to lie in the geometry of the amide bond between 3-phenyllactic acid and *N*-methyldehydroalanine. The absolute stereochemistery was elucidated by Marfey's analysis and chiral HPLC analysis of the acid hydrolysate of YM-254890.

1. Introduction

Platelets play a crucial role in thrombus formation, which causes various thromboembolic diseases such as unstable angina, myocardial infarction, stroke and peripheral arterial occlusive diseases.[1] In the course of our screening for platelet aggregation inhibitors, YM-254890 was found in the culture broth of *Chromobacterium* sp. QS3666.[2] It is well-established that ADP mediates platelet aggregation through two G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$. The $P2Y_1$ receptor is coupled to $G_{q/11}$ protein which is responsible for PLCβ activation followed by mobilization of intracellular calcium stores. The $P2Y_{12}$ receptor is coupled to G_i and is responsible for adenylyl cyclase inhibition. Our recent studies suggested that YM-254890 inhibited $G_{q/11}$ protein, thereby inhibiting the $P2Y_1$ receptor-signal transduction pathway, but had no effect on the $P2Y_{12}$ receptor-signal transduction pathway (Saito et al., submitted). To our knowledge, the inhibitor of $G_{q/11}$ protein has not been reported so far, whereas another G protein, G_i, had a specific inhibitor, pertussis toxin. We describe here the isolation and structure elucidation of the first $G_{q/11}$ inhibitor, YM-254890.

2. Results and discussion

YM-254890 (**1**) (Fig. 1) was first isolated from the fermentation broth (2.5 L) of *Chromobacterium* sp. QS3666 by bioassay-guided fractionation.[2] A large-scale purification was carried out for structure elucidation and pharmacological studies. The fermentation broth (80 L) was filtered, and the filtrate was subjected to HP-20 column chromatography. The column was washed with H_2O and MeOH/H_2O (4:6), and then eluted with MeOH. The MeOH eluate was partitioned between EtOAc and H_2O, and the EtOAc extract was purified by ODS flash chromatography, silica gel flash chromatography, preparative HPLC and crystallization to afford 1.2 g of **1**.

The positive and negative ion FABMS spectra showed $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ peaks at *m*/*z* 960, 982 and 958, respectively, indicating a molecular weight of 959 for **1**. The molecular formula of **1** was established as $C_{46}H_{69}N_7O_{15}$ by HRFABMS and NMR spectroscopy. The IR spectrum of **1** exhibited typical absorption bands of a peptide at 3340, 1680, 1640 and 1530 cm^{-1}. The amino acid analysis of the acid hydrolysate of **1** revealed the presence of Ala and Thr as common amino acids.

The ^1H NMR spectrum in DMSO-d_6 did not give sharp signals, whereas the ^1H NMR spectrum in $CDCl_3$ displayed a 10:6 mixture of signals, which we attributed to a mixture of two similar analogues or to the existence of two conformers. This feature persisted in such NMR solvents as CD_3OD, CD_3CN, acetone-d_6, dioxane-d_8, THF-d_8 and DMF-d_7 showing signals intensities ranging from 10:2 to 10:6. This observation indicated that **1** was existing as a mixture of two conformers. NMR analysis of the major conformer was carried out with NMR spectra in dioxane-d_8 which displayed a 10:2 mixture of signals. The ^1H NMR spectrum, showing five exchangeable NH or OH protons at

Keywords: YM-254890; cyclic peptide; depsipeptide; $G_{q/11}$ inhibitor.

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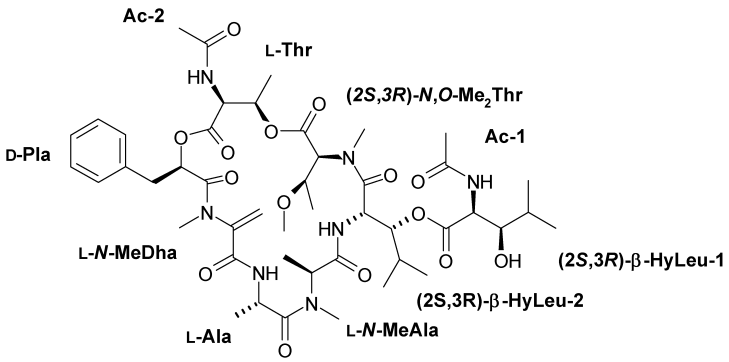

Figure 1. Structure of YM-254890 (**1**).

δ_H 6.75–8.36, four *N*-methyl or *O*-methyl groups at δ_H 2.62–3.37 and five aromatic protons at δ_H 7.19–7.26, implied that **1** is a highly methylated peptide containing both aliphatic and aromatic residues. The ^{13}C NMR and DEPT spectra revealed the presence of fourteen methyls, two methylenes (of which one is sp^2), eighteen methines (of which five are aromatic), two sp^2 quaternary carbons and ten carbonyls. Fifteen out of the sixteen degrees of unsaturation required by the molecular formula were accounted for by functional groups (one exomethylene, ten carbonyls and a monosubstituted benzene ring), suggesting the cyclic nature of **1**.

Analysis of one- and two-dimensional NMR spectra including COSY, HOHAHA, HMQC and HMBC led to the assignments of seven amino acids (β-hydroxyleucine-1 (β-HyLeu-1), β-hydroxyleucine-2 (β-HyLeu-2), *N*,*O*-dimethylthreonine (*N*,*O*-Me_2Thr), threonine (Thr), *N*-methyldehydroalanine (*N*-MeDha), alanine (Ala), and *N*-methylalanine (*N*-MeAla)), one α-hydroxy acid (3-phenyllactic acid (Pla)) and two acetyl groups (Table 1). Oxygenation of the β-carbons in β-HyLeu-1 and -2 was inferred from the chemical shifts at δ_C 78.7 and δ_C 77.2, respectively. The hydroxyl proton (δ_H 6.76) of β-HyLeu-1 was assigned by the COSY spectrum. The linkage between the α-carbon and β-carbon of β-HyLeu-2 was deduced from correlations in the HMBC spectrum (α-H/γ-C, β-H/CO), whereas the α-proton gave no cross peak with the β-proton in the COSY spectrum, indicating that the dihedral angle between the CH bonds at α-C and β-C was nearly 90°. The *N*,*O*-dimethyl groups of *N*,*O*-Me_2Thr were disclosed by HMBC correlations (α-H/NMe, NMe/α-C, β-H/OMe, OMe/β-C) and the chemical shifts of β-C (δ_C 72.9) and OMe (δ_C 57.2). In the Pla residue, the proton chemical shifts at δ_H 7.19–7.26 (monosubstituted benzene ring), 3.10 and 2.89 (methylene), and 5.19 (methine) are very similar to those reported for phenylalanine. However, the carbon chemical shift of the methine (δ_C 73.2) was that of an oxymethine, indicating that this residue is not phenylalanine but 3-phenyllactic acid. *N*-MeDha was deduced through analysis of HMBC correlations as follows: the exomethylene protons (δ_H 5.28 and 5.12) gave cross peaks with α-carbon (δ_C 147.0) and carbonyl (δ_C 164.0). HMBC correlations between the *N*-methyl protons (δ_H 3.21) and α-carbon (δ_C 164.0) established the presence of the *N*-methyl group in dehydroalanine.

The sequencing of these residues was accomplished by interpretation of HMBC and ROESY spectra (Fig. 2). The HMBC correlations from the NH protons or *N*-methyl protons to the neighboring carbonyl carbons were seen between *N*,*O*-Me_2Thr/β-HyLeu-2, β-HyLeu-2/*N*-MeAla, *N*-MeAla/Ala, Ala/*N*-MeDha, *N*-MeDha/Pla, β-HyLeu-1/Ac-1, and Thr/Ac-2. Correlations from the α-protons to the carbonyl carbons of the neighboring amino acids were also observed between *N*,*O*-Me_2Thr/β-HyLeu-2, β-HyLeu-2/*N*-MeAla, and *N*-MeAla/Ala. The two ester linkages were deduced from the HMBC correlations from the β-proton of β-HyLeu-2 to the carbonyl carbon of β-HyLeu-1 and from the β-proton of Thr to the carbonyl carbon of *N*,*O*-Me_2Thr. Considering the molecular formula, the hydroxyl group in Pla had to be combined with the carboxyl group in Thr via an ester bond. Thus, the planar structure of **1** was determined as shown in Figure 1.

^1H and ^{13}C NMR assignments for the major and minor conformers were elucidated by interpretation of the NMR spectra (in $CDCl_3$) which displayed a 10:6 mixture of signals (Table 2). In the major conformer, the ROESY correlations (α-H of β-HyLeu-2/α-H of *N*,*O*-Me_2Thr, α-H of Ala/α-H of *N*-MeAla; H-2 of Ac-1/NH of β-HyLeu-1, H-2 of Ac-2/NH of Thr, α-H of Pla/NMe of *N*-MeDha, β-Ha of *N*-MeDha/NH of Ala) indicated the presence of two *cis*-amide bonds (*N*,*O*-Me_2Thr/β-HyLeu-2, *N*-MeAla/Ala) and five *trans*-amide bonds (Ac-1/β-HyLeu-1, Ac-2/Thr, Pla/*N*-MeDha, *N*-MeDha/Ala). The same ROESY correlations were observed in the minor conformer, except that no ROESY correlation between the α-proton of Pla and the *N*-methyl protons of *N*-MeDha was observed. This indicated that the distinction between major and minor conformers may lie in the geometry of the amide bond between Pla and *N*-MeDha. The chemical shift of β-carbon of *N*-MeDha was much different between both conformers, implied that it was influenced by the steric hindrance caused by the geometry of the amide bond. In the minor conformer, the high-field chemical shift (δ_H 3.70) of β-Hb of *N*-MeDha suggested that the proton might sit just above/below the plane of the benzene ring of Pla and be affected by the anisotropic shielding effect.

The relative stereochemistry of β-HyLeu-1, β-HyLeu-2, Thr and *N*,*O*-Me_2Thr was deduced from interpretation of $^3J_{HH}$ values and ROESY correlations (Fig. 3).[3] Small vicinal coupling constants for α-H/β-H of β-HyLeu-1, -2 and Thr (2.0, 0 and 2.0 Hz, respectively) indicated restricted rotations and gauche relationships for these protons. In the

Table 1. ^{1}H and ^{13}C NMR data for the major conformer of YM-254890 (**1**) in dioxane-d_8

Residue	Position	^{13}C	^{1}H (mutt, J in Hz)	HMBC
Ac-1	1	170.6		
	2	22.1	2.12 (s)	C-1 (Ac-1)
β-HyLeu-1	α	57.6	4.39 (dd, 7.9, 2.0)	β-C, CO (β-HyLeu-1)
	β	78.7	3.67 (m)	
	γ	30.6	1.96 (m)	β-C (β-HyLeu-1)
	δ	a 20.6	a 1.16 (d, 6.3)	β-C, γ-C, δ-Cb (β-HyLeu-1)
		b 18.6	b 0.86 (d, 6.8)	β-C, γ-C, δ-Ca (β-HyLeu-1)
	CO	170.7		
	NH		7.17 (d, 7.9)	α-C, β-C (β-HyLeu-1), C-1 (Ac-1)
	β-OH		6.76 (d, 3.9)	β-C, γ-C (β-HyLeu-1)
β-HyLeu-2	α	47.5	5.35 (d, 9.5)	γ-C, CO (β-HyLeu-2), CO (*N*-MeAla)
	β	77.2	5.29 (d, 9.2)	γ-C, δ-Ca, δ-Cb, CO (β-HyLeu-2), CO (β-HyLeu-1)
	γ	31.3	1.73 (m)	β-C (β-HyLeu-2)
	δ	a 19.6	a 1.04 (d, 6.8)	β-C, γ-C, δ-Cb (β-HyLeu-2)
		b 18.3	b 0.78 (d, 6.8)	β-C, γ-C, δ-Ca (β-HyLeu-2)
	CO	172.2		
	NH		6.75 (d, 9.5)	CO (*N*-MeAla)
N,*O*-Me_2Thr	α	65.1	4.04 (d, 9.7)	β-C, γ-C, CO, NMe (N,O-Me_2Thr), CO (β-HyLeu-2)
	β	72.9	3.77 (m)	α-C, OMe (*N*,*O*-Me_2Thr)
	γ	16.5	1.16 (d, 6.3)	α-C, β-C (*N*,*O*-Me_2Thr)
	CO	167.0		
	NMe	29.1	2.62 (s)	α-C (*N*,*O*-Me_2Thr), CO (β-HyLeu-2)
	OMe	57.2	3.37 (s)	β-C (*N*,*O*-Me_2Thr)
Ac-2	1	170.9		
	2	22.2	2.14 (s)	C-1 (Ac-2)
Thr	α	53.2	5.04 (dd, 10.2, 2.0)	CO (Thr)
	β	70.6	5.38 (dq, 2.0, 6.3)	γ-C (Thr), CO (*N*,*O*-Me_2Thr)
	γ	16.6	1.11 (d, 6.3)	α-C, β-C (Thr)
	CO	169.9		
	NH		7.37 (d, 10.2)	α-C (Thr), CO (Ac-2)
Pla	1	168.7		
	2	73.2	5.19 (dd, 9.3, 3.4)	C-1, C-2, C-3 (Pla)
	3	36.9	a 3.10 (dd, 14.7, 3.4)	C-1, C-4, C-5/9 (Pla)
			b 2.89 (dd, 14.7, 9.3)	C-1, C-2, C-4, C-5/9 (Pla)
	4	137.4		
	5/9	130.5	7.25 (m)	C-3, C-7 (Pla)
	6/8	128.9	7.26 (m)	C-4, C-6/8 (Pla)
	7	127.4	7.19 (m)	C-5 (Pla)
N-MeDha	α	147.0		
	β	106.7	a 5.28 (d, 2.0)	α-C, CO (*N*-MeDha)
			b 5.12 (d, 2.0)	α-C, CO (*N*-MeDha)
	CO	164.0		
	NMe	36.3	3.21 (s)	α-C (*N*-MeDha), CO (Pla)
Ala	α	46.3	4.85 (m)	β-C, CO (Ala), CO (*N*-MeDha)
	β	18.5	1.30 (d, 6.3)	α-C, CO (Ala)
	CO	173.1		
	NH		8.36 (d, 9.2)	α-C (Ala), CO (*N*-MeDha)
N-MeAla	α	57.0	4.72 (q, 6.9)	β-C, CO, NMe (*N*-MeAla), CO (Ala)
	β	14.0	1.35 (d, 6.9)	α-C, CO (*N*-MeAla)
	CO	170.9		
	NMe	31.7	2.84 (s)	α-C (*N*-MeAla), CO (Ala)

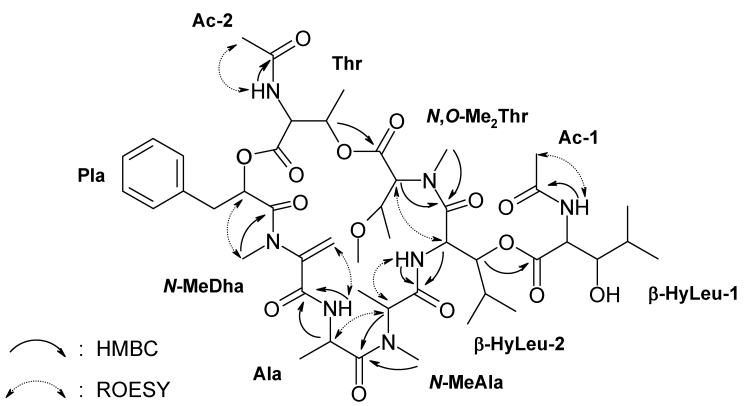

Figure 2. HMBC and ROESY correlations for the major conformer of YM-254890 (**1**).

Table 2. ^{1}H and ^{13}C NMR data for both conformers of YM-254890 (**1**) in $CDCl_3$

Residue	Position	Major conformer		Minor conformer	
		^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (multi, *J* in Hz)
Ac-1	1	171.1		171.1	
	2	22.4	2.25 (s)	22.4	2.24 (s)
β-HyLeu-1	α	57.0	4.54 (dd, 8.0, 2.0)	56.9	4.60 (dd, 8.0, 1.1)
	β	78.2	3.75 (m)	78.6	3.75 (m)
	γ	30.1	1.98 (m)	30.1	1.98 (m)
	δ	a 20.5	a 1.18 (d, 6.3)	a 20.4	a 1.10 (d, 6.5)
		b 18.6	b 0.89 (d, 6.8)	b 18.7	b 0.92 (d, 7.3)
	CO	170.2		170.6	
	NH		7.26 (d, 8.0)		7.37 (d, 8.0)
	β-OH		6.84 (d, 4.4)		6.32 (d, 4.4)
β-HyLeu-2	α	46.8	5.36 (d, 9.7)	46.5	5.57 (d, 9.8)
	β	77.1	5.32 (d, 10.3)	78.7	5.11 (m)
	γ	30.6	1.73 (m)	30.0	1.71 (m)
	δ	a 19.5	a 1.10 (d, 7.0)	a 19.4	a 1.14 (d, 6.0)
		b 18.2	b 0.84 (d, 6.8)	b 18.4	b 0.84 (d, 6.8)
	CO	171.3		172.6	
	NH		6.77 (d, 9.7)		6.66 (d, 9.8)
N,*O*-Me_2Thr	α	64.4	4.05 (d, 10.3)	64.8	4.20 (d, 9.8)
	β	72.3	3.77 (m)	71.7	3.85 (m)
	γ	16.4	1.19 (d, 5.4)	16.4	1.26 (d, 5.9)
	CO	166.3		166.1	
	NMe	28.9	2.68 (s)	28.9	2.93 (s)
	OMe	57.1	3.41 (s)	57.0	3.45 (s)
Ac-2	1	171.6		170.6	
	2	22.4	2.27 (s)	22.6	2.14 (s)
Thr	α	52.8	5.02 (dd, 9.8, 1.4)	54.9	5.01 (dd, 8.8, 1.9)
	β	69.6	5.50 (m)	75.0	5.35 (m)
	γ	16.7	1.22 (d, 6.8)	16.5	1.32 (d, 5.8)
	CO	169.0		167.9	
	NH		7.64 (d, 9.8)		7.64 (d, 8.8)
Pla	1	167.9		167.7	
	2	72.7	5.21 (dd, 8.8, 3.9)	70.7	5.10 (dd, 8.5, 4.4)
	3	36.6	a 3.12 (dd, 15.2, 3.9)	38.4	a 3.33 (dd, 12.7, 8.5)
			b 2.98 (dd, 15.2, 8.8)		b 2.78 (dd, 12.7, 4.4)
	4	136.0		134.6	
	5/9	129.5	7.24 (m)	130.0	7.18 (m)
	6/8	128.6	7.28 (m)	128.8	7.18 (m)
	7	127.0	7.24 (m)	127.8	7.28 (m)
N-MeDha	α	145.5		141.5	
	β	107.0	a 5.34 (d, 1.5)	122.6	a 5.70 (d, 1.5)
			b 5.09 (d, 1.5)		b 3.70 (d, 1.5)
	CO	164.0		162.1	
	NMe	36.3	3.19 (s)	37.5	3.03 (s)
Ala	α	45.9	4.91 (m)	46.1	5.10 (m)
	β	18.0	1.40 (d, 6.8)	18.9	1.30 (d, 5.4)
	CO	172.7		172.4	
	NH		8.51 (d, 9.3)		7.70 (d, 9.7)
N-MeAla	α	56.5	4.76 (q, 6.8)	56.0	4.80 (q, 6.3)
	β	14.2	1.40 (d, 6.8)	14.0	1.49 (d, 6.8)
	CO	170.0		171.0	
	NMe	31.5	2.90 (s)	31.1	2.88 (s)

ROESY spectrum, the α-, β-, γ-, and OH protons of β-HyLeu-1 were correlated to the γ-, α- and NH protons of β-HyLeu-1 and the β-proton of β-HyLeu-2, respectively, suggesting *threo* configuration of β-HyLeu-1. The configurations of β-HyLeu-2 and Thr were also determined as *threo* by the ROESY correlations. In the *N*,*O*-Me_2Thr, a large coupling constant for α-H/β-H (9.7 Hz) and the ROESY correlations (α-H/γ-H, β-H/NMe, NMe/OMe) indicated *anti* arrangement for these protons and *threo* configuration of this residue.

The absolute stereochemistry of each amino acid was determined by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, FDAA).[4] HPLC analysis of the derivative eluted by linear gradient of MeCN/H_2O (0.05% TFA) revealed the presence of L-Ala, L-Thr, (2*S*,3*R*)-*N*,*O*-Me_2Thr and (2*S*,3*R*)-β-HyLeu, whereas the configurations of L- and D-*N*-MeAla could not be distinguished because of their identical retention times. After trying a variety of HPLC conditions, HPLC analysis using MeOH/H_2O (50:50, 0.05% TFA) as an eluent enabled us to determine the chirality of *N*-MeAla as L. The absolute configuration of Pla was determined to be D by chiral HPLC analysis. Thus, the absolute stereochemistry of **1** was determined as shown in Figure 1.

YM-254890, the first $G_{q/11}$ inhibitor, is a cyclic depsipeptide

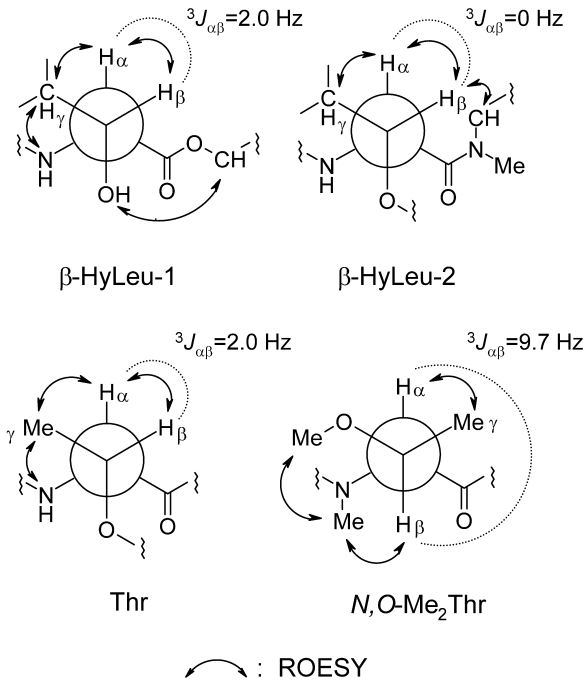

Figure 3. Relative stereochemistry of β-HyLeu-1, β-HyLeu-2, Thr and *N*,*O*-Me_2Thr from ROESY and $^3J_{HH}$ data.

containing uncommon amino acids, β-hydroxyleucine (two residues), *N*,*O*-dimethylthreonine and *N*-methyldehydroalanine. To our knowledge, there is no compound similar to YM-254890 among the known peptides of bacterial origin, although the cyclic depsipeptide FR900359, which was isolated from the whole plant of *Ardisa crenata sims*, is similar.[5] FR900359 differs from YM-254890 in an amino acid constituent and an acyl group. Structure elucidation of FR900359 was carried out by chemical studies and X-ray crystallographic analysis. Hence, this is the first report wherein the ^{1}H and ^{13}C NMR data of this class of peptide are fully and unambiguously assigned by detailed examination of one- and two-dimensional NMR spectra. The absolute stereochemistry of YM-254890 was determined by Marfey's analysis and chiral HPLC analysis of the acid hydrolysate, and was found to be identical to that of FR900359, which was determined by X-ray crystallographic analysis and chiral HPLC analysis.[6] YM-254890 inhibited ADP-induced platelet aggregation in human platelet-rich plasma with an IC_{50} value of 0.39 μM.[2] The ratio between major and minor conformers of YM-254890 in the assay buffer could not be measured because of the low solubility in water, and stucture–activity relationship of both conformers has not been determined. YM-254890 had no cytotoxicity against MG63 human osteosarcoma cells even at 30 μM, while FR900359 was reported to inhibit platelet aggregation and have cytotoxicity against rat fibroblasts and myelocytic leukemia cells. Further pharmacological studies are now underway.

3. Experimental

3.1. General procedures

Reagents and solvents were obtained from commercial suppliers and used as received. β-Hydroxyluecine (2-amino-3-hydroxy-4-methylpentanoic acid) was obtained from Kanto Chemicals. FABMS and HRFABMS spectra were measured on JEOL DX-300 and JMS-700T mass spectrometers, respectively. Optical rotations were determined using a HORIBA SEPA-200 polarimeter. UV and IR spectra were recorded on Shimadzu UV-2200 and Perkin–Elmer 2000 FT-IR spectrophotometers, respectively. NMR spectra were recorded on a JNM-A500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, using TMS as an internal standard. HMQC experiments were optimized for $^1J_{CH}$=145 Hz and HMBC experiments for $^nJ_{CH}$=8 Hz.

3.2. Fermentation and isolation

Chromobacterium sp. QS3666 was inoculated into each of sixteen 500 mL Erlenmeyer flasks containing 100 mL of a seed medium consisting of glucose 1.0%, potato starch 2.0%, Polypepton (Nihon Pharmaceutical Co., Ltd) 0.5%, yeast extract 0.5%, CaCO3 0.4% (pH 7.0). After incubation at 28°C for three days on a rotary shaker at 220 rpm, the seed culture was inoculated into each of four 30 L jar fermentors containing 20 L of a production medium consisting of glycerol 2.0%, glucose 0.5%, Polypepton 0.5%, meat extract 0.5%, yeast extract 0.1%, NaCl 0.1%, antifoam 0.05% (pH6.5). Fermentation was carried out at 24°C for three days under agitation of 400 rpm and aeration of 1 vvm. The fermentation broth (80 L) was filtered, and the filtrate adjusted to pH 4.0 and was subjected to HP-20 column chromatography (DIAION HP-20, 102×970 mm, Mitsubishi Chemical). The column was washed with H_2O and MeOH/H_2O (4:6), and then eluted with MeOH. The MeOH eluate was partitioned between EtOAc and H_2O. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown extract (22.9 g). This oily extract was subjected to ODS flash chromatography (YMC-GEL ODS-A 120–130/70, 100×60 mm, YMC) and eluted with a step gradient from 60% to 100% MeOH. The fractions eluted with 80% and 90% MeOH were combined and concentrated to dryness *in vacuo* (9.5 g). The yellow residue was subjected to silica gel flash chromatography (Kieselgel 60 0.040–0.063 mm, 60×130 mm, Merck), with elution by a step gradient of $CHCl_3$–MeOH solvent system. The fractions eluted with $CHCl_3$–MeOH (100:0 and 98:2) were evaporated to dryness (3.1 g) and the residue was repeatedly purified by preparative HPLC (CAPCELL PAK C18 UG120, 20×250 mm, Shiseido) with MeOH/H_2O (75:25) at a flow rate of 8 mL/min. The fractions that eluted at 18.4 min were combined and evaporated to dryness (1.3 g), and the residue was crystallized from *n*-hexane to yield 1.2 g of YM-254890 as a white crystalline solid.

3.2.1. YM-254890 (1). $[\alpha]_D^{25}=-75.8°$ (c 0.30, MeOH); UV (MeOH) λ_{max} nm (ϵ) 203 (31100); IR ν_{max} (KBr) 3430, 3340, 2980, 2940, 1750, 1680, 1640, 1530, 1460, 1410, 1370, 1280, 1230, 1170, 1100, 1080 cm^{-1}; positive FABMS *m*/*z* 960 $[M+H]^+$, *m*/*z* 982 $[M+Na]^+$; negative FABMS *m*/*z* 958 $[M-H]^-$; HRFABMS *m*/*z* 960.4938 $[M+H]^+$, Calcd for $C_{46}H_{70}N_7O_{15}$, Δ+0.8 mmu; ^{1}H and ^{13}C NMR are listed in Tables 1 and 2.

3.3. Amino acid analysis

YM-254890 (0.5 mg) in 0.5 mL of 6N HCl was heated in a sealed tube at 110°C for 16 h. After evaporation, the residual hydrolysate was suspended in 50 μl of 0.1 M borate buffer and treated with 50 μl of 25 mM NBD-F (7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) solution in ethanol, and heated at 60°C for 5 min.[7] The reaction mixture was analyzed by HPLC on TSKgel ODS 80Ts (4.6×150 mm, TOSOH) at a flow rate of 1 mL/min with UV detection at 470 nm, using the following gradient condition: 10 mM citrate buffer (pH 6.2)/50% MeCN, from 94:6 to 64:36 for the first 16 min, then from 64:36 to 63:37 for the next 5 min, and finally from 63:37 to 0:100 for the last 14 min. The NBD derivative of the acid hydrolysate was identified by comparing the retention times with similarly derivatized authentic standards.

3.4. Marfey analysis of amino acids

YM-254890 (3 mg) in 1 mL of 6N HCl was heated in a sealed tube at 110°C for 14 h, and the reaction mixture extracted with EtOAc (1 mL×3). The aqueous layer was dried under Ar, and the residue dissolved in 200 μl of water. Subsequently, 20 μl of this solution was treated with 40 μl of 1% FDAA solution in acetone and 8.5 μl of 1 M $NaHCO_3$, and heated at 40°C for 1 h. After cooling to room temperature, 20 μl of 1N HCl was added and the solution was analyzed by HPLC on a Cadenza CD-C18 (4.6×250 mm, Imtakt) at a flow rate of 1 mL/min with UV detection at 340 nm, using a linear gradient from MeCN/H_2O (20:80, 0.05% TFA) to MeCN/H_2O (45:55, 0.05% TFA) over 30 min. The FDAA derivative of the acid hydrolysate was identified by comparing the retention times with those of authentic standards. The retention times of the FDAA derivative of the acid hydrolysate were as follows: L-Ala 16.8 min (D-Ala 19.9 min), L-Thr 12.5 min (D-Thr 15.7 min, L-allo-Thr 12.7 min, D-allo-Thr 14.0 min), L and D-*N*-MeAla 19.0 min, (2*S*,3*R*)-β-HyLeu 20.0 min ((2*R*,3*S*)-β-HyLeu 26.1 min), and (2*S*,3*R*)-*N*,*O*-Me_2Thr 21.7 min ((2*R*,3*S*)-*N*,*O*-Me_2Thr 22.9 min). The configuration of *N*-MeAla could not be determined by the solvent system described above. The isocratic solvent system of MeOH/H_2O (50:50, 0.05% TFA) was used to separate the FDAA derivatives of L and D-*N*-MeAla (L 14.8 min, D 14.3 min).

3.5. Chiral HPLC analysis

YM-254890 (3 mg) in 1 mL of 6N HCl was heated in a sealed tube at 110°C for 14 h, and the reaction mixture was extracted with EtOAc (1 mL×3). The organic layer was dried under Ar, and the residue dissolved in 200 μl of MeOH. The hydrolysate was analyzed by chiral HPLC on SUMICHIRAL OA-5000 (4.6×150 mm, Sumika Chemical) at a flow rate of 2 mL/min with UV detection at 254 nm, using 2.0 mM $CuSO_4$ in 2-propanol/H_2O (15:85) as an eluent. The retention time of the acid hydrolysate was 43.0 min (D-Pla; 31.7 min L-Pla).

3.6. Synthesis of (2*S*,3*R*)-*N*,*O*-Me_2Thr and (2*R*,3*S*)-*N*,*O*-Me_2Thr

Boc-L-Thr (500 mg, 2.28 mmol) and iodomethane (1.42 mL, 10 equiv.) were dissolved in dry THF (10 mL) and cooled to 0°C. Sodium hydride (60% dispersion in paraffin liquid, 456 mg, 5 equiv.) was added to the solution with vigorous stirring. After stirring at room temperature for 17 h, saturated aqueous NH_4Cl (10 mL) was added and the mixture extracted with diethyl ether (10 mL). The organic layer was extracted with saturated aqueous $NaHCO_3$/water (1:1) (3×5 mL). The aqueous layers were combined, adjusted to pH 4 with 10% citric acid, and extracted with EtOAc (2×10 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to give Boc-(2*S*,3*R*)-*N*,*O*-Me_2Thr as an oil (427 mg, 76%). 4N HCl in EtOAc (2 mL) was added to a solution of Boc-(2*S*,3*R*)-*N*,*O*-Me_2Thr (170 mg, 0.69 mmol) in MeOH (2 mL). The mixture was stirred at room temperature for 1 h, and evaporated to give (2*S*,3*R*)-*N*,*O*-Me_2Thr as an oil (124 mg, 98%). ^{1}H NMR (300 MHz, D_2O) δ 1.18 (3H, d, *J*=6.2 Hz), 2.62 (3H, s), 3.25 (3H, s), 3.64 (1H, d, *J*=6.6 Hz), 3.72 (1H, m); ESI-MS *m*/*z* 148 $[M+H]^+$. Identical ^{1}H NMR and ESI-MS spectra were exhibited by (2*R*,3*S*)-*N*,*O*-Me_2Thr, which was synthesized from Boc-D-Thr by the same procedure.

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