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YM-254890 analogues, novel cyclic depsipeptides with $G\alpha_{q/11}$ inhibitory activity from *Chromobacterium* sp. QS3666

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Abstract—The structure elucidation and biological activity of novel YM-254890 (**1**) analogues and semi-synthetic derivatives are described. Three natural analogues, YM-254891 (**2**), YM-254892 (**3**), and YM-280193 (**4**), were isolated from the fermentation broth of *Chromobacterium* sp. QS3666, and two hydrogenated derivatives, YM-385780 (**5**) and YM-385781 (**6**), were synthesized from YM-254890. Their structures were determined by one- and two-dimensional NMR studies and mass spectrometry. Among these compounds, two natural analogues **2**–**3** which possessed acyl groups at β-HyLeu-1 and one derivative **6** whose conformation was similar to that of **1** showed comparable $G\alpha_{q/11}$ inhibitory activity to that of **1**. This indicates that the acyl β-HyLeu residue plays an important role in activity and also that the α,β-unsaturated carbonyl group of the *N*-MeDha residue is not critical to activity. The other hydrogenated derivative **5** had significantly less activity, which could be attributed to conformational differences.

1. Introduction

Heterotrimeric GTP-binding proteins (G proteins) transduce signals from various cell surface receptors (G protein-coupled receptors, GPCRs) to intracellular effectors.[1] Ligand-bound receptors catalyze the exchange of GDP for GTP on the α subunit of the G protein, resulting in dissociation of the α subunit from the βγ subunits. The GTP-bound α subunit then modulates the intracellular effectors. Signaling is terminated by hydrolysis of the bound GTP to GDP by the intrinsic GTPase activity of the α subunit. There are four families of G proteins, Gs, Gi, Gq, and G12, which are classified based on sequence and functional similarities of the α subunit. The α subunit of the Gs protein predominantly activates adenylyl cyclase. The α subunit of the Gi protein can inhibit adenylyl cyclase or regulate ion channel activity, while the α subunit of the Gq protein can stimulate phospholipase Cβ[2] and the α subunit of the G12 protein can activate Bruton's tyrosine kinase or GAP. Among the four G protein families, only the Gi protein is known to be inhibited by Pertussis toxin, which is an invaluable probe in the analysis of the relationship between GPCR and intracellular signal transduction. Blockade of ligand-stimulated cellular response by Pertussis toxin can indicate that signal transduction is mediated by the Gi protein.

In the course of our screening for a platelet aggregation inhibitor, YM-254890 was found in the culture broth of *Chromobacterium* sp. QS3666.[3–5] YM-254890 strongly inhibited ADP-induced platelet aggregation by blockade of the $P2Y_1$ receptor-signal transduction pathway. YM-254890 exhibited dose-dependent inhibition of thrombus formation in an $FeCl_3$-induced carotid artery thrombosis model after intravenous bolus injection or oral administration in mice. Oral administration of YM-254890 inhibited neointima formation 3 weeks after vascular injury with significance at 1 mg/kg twice daily for 7 days. Interestingly, our recent studies suggested that YM-254890 is the first $G\alpha_{q/11}$ inhibitor, which blocks the exchange of GDP for GTP (Takasaki et al. submitted). YM-254890 directly inhibited the $G\alpha_{q/11}$ protein, thereby inhibiting the $P2Y_1$ receptor-signal transduction pathway and ADP-induced platelet aggregation. YM-254890 may be a valuable tool in achieving an enhanced understanding of the biological

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functions of $G\alpha_{q/11}$ protein-coupled signal transduction as well as a therapeutic agent to treat $G\alpha_{q/11}$-mediated diseases including thromboembolic diseases.

In order to study the structure–activity relationship, we examined the fermentation broth of *Chromobacterium* sp. QS3666 for natural analogues of YM-254890 (**1**) and identified a further three novel YM-254890 analogues, YM-254891 (**2**), YM-254892 (**3**), and YM-280193 (**4**). In addition, two hydrogenated derivatives, YM-385780 (**5**) and YM-385781 (**6**) were synthesized from YM-254890. We describe herein the structure elucidation and biological activity of both the natural analogues and the two semi-synthetic derivatives.

2. Results and discussion

YM-254891 (**2**), YM-254892 (**3**), and YM-280193 (**4**) were isolated from the fermentation broth (80 L) of *Chromobacterium* sp. QS3666. The fermentation broth 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 and preparative HPLC to afford **2** (287 mg), **3** (36 mg), and **4** (35 mg).

The molecular formula of YM-254891 (**2**) was determined to be $C_{47}H_{71}N_7O_{15}$ by high-resolution FABMS and NMR spectroscopy, which differed from YM-254890 (**1**) by the presence of an extra methylene. The ^{1}H NMR spectrum of **2** in dioxane-d_8 indicated a 10:2 mixture of two conformers, and was similar to that of **1** apart from the fact that the resonances for an acetyl group were notably absent while resonances for a propionyl group were detected. Analysis of one- and two-dimensional NMR spectra including COSY, HMQC, and HMBC led to the assignment of seven amino acids (β-hydroxyleucine-1 (β-HyLeu-1), β-hydroxyleucine-2 (β-HyLeu-2), *N*,*O*-dimethylthreonine (*N*,*O*-Me$_2$Thr), threonine (Thr), *N*-methyldehydroalanine (*N*-MeDha), alanine (Ala), and *N*-methylalanine (*N*-MeAla)), a 3-phenyllactic acid (Pla), and two acyl groups (an acetyl and a propionyl group) (Table 1). The sequencing of these residues was accomplished by interpretation of HMBC and ROESY spectra as shown in Figure 1. The chemical shifts, coupling constants and optical rotations of **1** and **2** were almost identical except for the acyl group, suggesting that the absolute stereochemistry of **2** was the same as **1**. Furthermore, the absolute stereochemistry was confirmed by Marfey's method with LC/MS[6] and chiral HPLC analysis of the acid hydrolysate of **2**. Therefore, **2** proved to be a novel YM-254890 analogue possessing a propionyl group at β-HyLeu-1.

The molecular formula of YM-254892 (**3**) was determined to be $C_{47}H_{71}N_7O_{15}S$ by high-resolution FABMS and NMR spectroscopy, which was 46 mass units larger than that of **1**. The ^{1}H NMR spectrum of **3** in dioxane-d_8 indicated a 10:2 mixture of two conformers similar to that observed for compounds **1** and **2**. The NMR spectra, including COSY, HMQC, and HMBC, were analyzed for the major conformer, and seven amino acids (β-HyLeu-1, β-HyLeu-2, *N*,*O*-Me$_2$Thr, Thr, *N*-MeDha, Ala, and *N*-MeAla) and a 3-phenyllactic acid (Pla) were assigned together with an acetyl and methylthioacetyl group (Table 1). The methylthioacetyl group was revealed by HMBC correlations (SMe/C-2, H-2/SMe, H-2/C-1) and the chemical shifts of the *S*-methyl group (δ_C 16.0 and δ_H 2.21). The sequencing of these residues was accomplished by interpretation of HMBC and ROESY spectra, as shown in Figure 1. The absolute stereochemistry of **3** was established to be identical to that of **1** due to the similarity between chemical shifts, coupling constants and optical rotations, which was confirmed by Marfey's method with LC/MS and chiral HPLC analysis of the acid hydrolysate. Thus, it was concluded that **3** was a novel YM-254890 analogue having a methylthioacetyl group at β-HyLeu-1. There are only a few natural products to our knowledge that possess sulfur-containing acyl groups,[7,8] and **3** is the first compound isolated from natural sources to contain a methylthioacetyl group.

The molecular formula of YM-280193 (**4**) was identified as $C_{38}H_{56}N_6O_{12}$ by high-resolution FABMS and NMR spectroscopy. Unlike **1**, **2**, and **3**, the ^{1}H NMR spectra of **4** in dioxane-d_8 and $CDCl_3$ were complex due to a 10:9 mixture of two conformers. NMR analysis of the major conformer was performed with NMR spectra in CD_3CN, which displayed a 10:2 mixture of conformers, so that six amino acids (β-HyLeu, *N*,*O*-Me$_2$Thr, Thr, *N*-MeDha, Ala, and *N*-MeAla), a 3-phenyllactic acid (Pla), and an acetyl group could be assigned (Table 1). The sequencing of these residues was achieved by the interpretation of HMBC and ROESY spectra as shown in Figure 1. The absolute stereochemistry was elucidated by Marfey's method with LC/MS and chiral HPLC analysis of the acid hydrolysate. Therefore, **4** was identified as a novel YM-254890 analogue, which did not contain an acetyl β-HyLeu residue.

It is generally known that nucleophiles may attack α,β-unsaturated carbonyl groups via Michael addition and so it is conceivable that the $G\alpha_{q/11}$ inhibitory activity of **1** might be caused by Michael addition of a nucleophilic residue in the $G\alpha_{q/11}$ protein to the *N*-MeDha residue of **1**. To test whether *N*-MeDha was essential for activity, YM-254890 was hydrogenated to provide two diastereomers, YM-385780 (**5**) and YM-385781 (**6**). The ^{1}H NMR spectra of **5** and **6** in dioxane-d_8 showed a 10:8 and 10:9 mixture of conformers, respectively, and those in $CDCl_3$ displayed a 10:5 and 10:6 mixture of conformers, respectively. ^{1}H and ^{13}C NMR assignments for the major and minor conformers of **5** and **6** were elucidated by interpretation of the NMR spectra in $CDCl_3$ (Table 2). Application of Marfey's analysis with LC/MS enabled the determination of the absolute configurations of *N*-MeAla-1, as shown in Figure 1.

The *cis–trans* conformations of the amide bonds were determined by interpretation of ROESY and NOESY spectra. In the major conformers of **1**, **2**, and **3** in

Table 1. ^{1}H and ^{13}C NMR data for the major conformers of YM-254890 (**1**), YM-254891 (**2**), YM-254892 (**3**), and YM-280193 (**4**)

Residue	Position	YM-254890 (**1**) in dioxane-d_8		YM-254891 (**2**) in dioxane-d_8		YM-254892 (**3**) in dioxane-d_8		YM-280193 (**4**) in CD_3CN	
		^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)
Acetyl/propionyl/	1	170.6		174.3		170.5			
methylthioacetyl	2	22.1	2.12 (s)	28.7	2.47 (q, 7.5)	36.0	3.39 (s)		
	3			10.1	1.10 (t, 7.5)				
	SMe					16.0	2.21 (s)		
β-HyLeu-1	α	57.6	4.39 (dd, 7.9, 2.0)	57.5	4.40 (dd, 7.8, 1.9)	57.4	4.47 (dd, 8.3, 1.0)		
	β	78.7	3.67 (m)	78.7	3.67 (m)	78.9	3.67 (m)		
	γ	30.6	1.96 (m)	30.6	1.95 (m)	30.7	1.96 (m)		
	δ	a 20.6	a 1.16 (d, 6.3)	a 20.7	a 1.16 (d, 5.9)	a 20.6	a 1.16 (d, 5.9)		
		b 18.6	b 0.86 (d, 6.8)	b 18.6	b 0.86 (d, 6.7)	b 18.6	b 0.87 (d, 6.9)		
	CO	170.7		170.8		170.5			
	NH		7.17 (d, 7.9)		7.12 (d,7.8)		7.25 (d, 8.3)		
	β-OH		6.76 (d, 3.9)		6.77 (d, 4.3)		6.79 (d, 4.3)		
β-HyLeu-2	α	47.5	5.35 (d, 9.5)	47.4	5.35 (d, 9.9)	47.4	5.36 (d, 9.8)	51.3	4.93 (dd, 10.1, 7.0)
	β	77.2	5.29 (d, 9.2)	77.2	5.29 (d, 9.4)	77.6	5.30 (d, 10.2)	77.3	3.45 (m)
	γ	31.3	1.73 (m)	31.2	1.72 (m)	31.1	1.78 (m)	29.5	1.94 (m)
	δ	a 19.6	a 1.04 (d, 6.8)	a 19.6	a 1.04 (d, 6.4)	a 19.6	a 1.05 (d, 7.3)	a 21.0	1.01 (d, 7.0)
		b 18.3	b 0.78 (d, 6.8)	b 18.3	b 0.78 (d, 6.7)	b 18.5	b 0.82 (d, 6.4)	b 16.1	0.93 (d, 6.7)
	CO	172.2		172.2		172.3		171.0	
	NH		6.75 (d, 9.5)		6.75 (d, 9.9)		6.72 (d, 9.8)		7.00 (d, 10.1)
N,*O*-Me_2Thr	α	65.1	4.04 (d, 9.7)	65.1	4.04 (d, 9.9)	65.2	4.05 (d, 9.8)	69.0	3.45 (d, 8.9)
	β	72.9	3.77 (m)	72.9	3.77 (m)	72.9	3.78 (m)	75.0	3.90 (dq, 8.9, 5.8)
	γ	16.5	1.16 (d, 6.3)	16.5	1.16 (d, 5.9)	16.5	1.16 (d, 5.9)	18.9	1.31 (d, 5.8)
	CO	167.0		167.0		167.0		168.5	
	NMe	29.1	2.62 (s)	29.1	2.62 (s)	29.1	2.62 (s)	40.7	3.21 (s)
	OMe	57.2	3.37 (s)	57.2	3.38 (s)	57.2	3.37 (s)	57.2	3.26 (s)
Acetyl	1	170.9		170.8		170.8		171.4	
	2	22.2	2.14 (s)	22.2	2.14 (s)	22.2	2.14 (s)	23.0	2.05 (s)
Thr	α	53.2	5.04 (dd, 10.2, 2.0)	53.2	5.04 (dd, 10.0, 1.6)	53.2	5.04 (d, 9.8, 1.0)	56.2	4.78 (dd, 9.5, 2.4)
	β	70.6	5.38 (dq, 2.0, 6.3)	70.5	5.38 (dq, 6.4, 1.6)	70.6	5.38 (m)	71.0	5.79 (m)
	γ	16.6	1.11 (d, 6.3)	16.6	1.11 (d, 6.4)	16.6	1.11 (d, 6.3)	17.1	1.15 (d, 6.4)
	CO	169.9		169.9		169.9		170.2	
	NH		7.37 (d, 10.2)		7.38 (d, 10.0)		7.38 (d, 9.8)		6.80 (d, 9.5)
Pla	1	168.7		168.7		168.7		169.1	
	2	73.2	5.19 (dd, 9.3, 3.4)	73.1	5. 1 9 (dd, 9.1, 3.7)	73.1	5.19 (dd, 9.0, 3.4)	72.1	5.55 (dd, 10.2, 5.4)
	3	36.9	a 3.10 (dd, 14.7,3.4)	36.9	a 3.10 (dd, 14.5, 3.7)	36.9	a 3.10 (dd, 14.6, 3.4)	39.3	3.12 (m)
			b 2.89 (dd, 14.7, 9.3)		b 2.89 (dd, 14.5, 9.1)		b 2.89 (dd, 14.6, 9.0)		
	4	137.4		137.4		137.4		136.6	
	5/9	130.5	7.25 (m)	130.5	7.25 (m)	130.5	7.26 (m)	131.2	7.24 (m)
	6/8	128.9	7.26 (m)	128.9	7.26 (m)	128.9	7.27 (m)	129.5	7.28 (m)
	7	127.4	7.19 (m)	127.3	7.18 (m)	127.4	7.19 (m)	128.1	7.28 (m)
N-MeDha	α	147.0		147.0		147.0		142.9	
	β	106.7	a 5.28 (d, 2.0)	106.6	a 5.28 (d, 1.7)	106.6	a 5.29 (d, 1.5)	123.1	a 5.65 (br s)

Table 1 (*continued*)

Residue	Position	YM-254890 (**1**) in dioxane-d_8		YM-254891 (**2**) in dioxane-d_8		YM-254892 (**3**) in dioxane-d_8		YM-280193 (**4**) in CD_3CN	
		^{13}C	^1H (mult., J in Hz)	^{13}C	^1H (mult., J in Hz)	^{13}C	^1H (mult., J in Hz)	^{13}C	^1H (mult., J in Hz)
			b 5.12 (d, 2.0)		b 5.12 (d, 1.7)		b 5.12 (d, 1.5)		b 3.77 (br s)
	CO	164.0		164.0		164.0		162.8	
	NMe	36.3	3.21 (s)	36.3	3.21 (s)	36.3	3.21 (s)	37.3	2.90 (s)
Ala	α	46.3	4.85 (m)	46.2	4.85 (m)	46.2	4.86 (m)	45.0	5.12 (dq, 7.8, 6.7)
	β	18.5	1.30 (d, 6.3)	18.5	1.30 (d, 6.4)	18.5	1.30 (d, 6.8)	17.5	1.21 (d, 6.7)
	CO	173.1		173.0		173.2		173.3	
	NH		8.36 (d, 9.2)		8.37 (d, 9.1)		8.34 (d, 9.3)		7.10 (d, 7.8)
N-MeAla	α	57.0	4.72 (q, 6.9)	57.0	4.72 (q, 6.7)	57.1	4.71 (q, 6.9)	62.1	3.66 (q, 7.0)
	β	14.0	1.35 (d,6.9)	14.0	1.35 (d, 7.0)	14.0	1.35 (d, 6.9)	13.1	1.40 (d, 7.0)
	CO	170.9		170.8		170.9		170.3	
	NMe	31.7	2.84 (s)	31.7	2.83 (s)	32.1	2.85 (s)	38.8	3.20 (s)

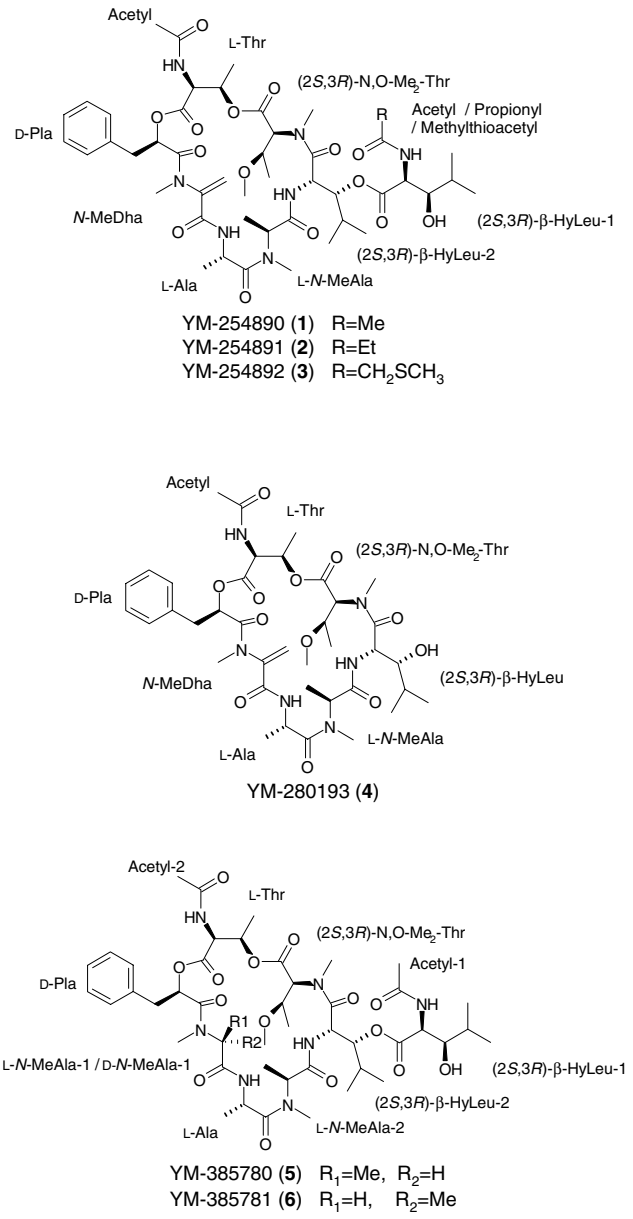

Figure 1. Structures of YM-254890 analogues and semi-synthetic derivatives (**1**–**6**).

dioxane-d_8, the ROESY correlations (α-H of β-HyLeu-2/α-H of *N*,*O*-Me_2 Thr, α-H of Ala/α-H of *N*-MeAla; H-2 of acetyl (propionyl, methylthioacetyl)/NH of β-HyLeu-1, H-2 of acetyl/NH of Thr, α-H of Pla/NMe of *N*-MeDha, β-Ha of *N*-MeDha/NH of Ala, α-H of *N*-MeAla/NH of β-HyLeu-2) indicated the presence of two *cis*-amide bonds (β-HyLeu-2/*N*,*O*-Me_2Thr, Ala/*N*-MeAla) and five *trans*-amide bonds (acetyl (propionyl, methylthioacetyl)/β-HyLeu-1, acetyl/Thr, Pla/*N*-MeDha, *N*-MeDha/Ala, *N*-MeAla/β-HyLeu-2). The similarity in the ROESY correlations, chemical shifts, and coupling constants suggested that the structural differences among **1**, **2**, and **3** did not affect conformation. On the other hand, the conformations of all the amide bonds of **4** in CD_3CN were determined to be *trans* apart from the Pla/*N*-MeDha amide bond by NOESY correlations (H-2 of acetyl/NH of Thr, β-Ha of *N*-MeDha/

Table 2. ^{1}H and ^{13}C NMR data for the major and minor conformers of YM-254890 (**1**), YM-385780 (**5**), and YM-385781 (**6**)

Residue	Position	YM-254890 (**1**) in $CDCl_3$				YM-385780 (**5**) in $CDCl_3$				YM-385781 (**6**) in $CDCl_3$			
		Major conformer		Minor conformer		Major conformer		Minor conformer		Major conformer		Minor conformer	
		^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)
Acetyl-1	1	171.1		171.1		171.2		171.2		171.1		171.1	
	2	22.4	2.25 (s)	22.4	2.24 (s)	22.5	2.10 (s)	22.4	2.24 (s)	22.4	2.24 (s)	22.4	2.25 (s)
β-HyLeu-1	α	57.0	4.54 (dd, 8.0, 2.0)	56.9	4.60 (dd, 8.0, 1.1)	55.5	4.83 (d, 9.8)	57.2	4.50 (br d, 6.1)	56.9	4.63 (dd, 7.9, 1.8)	57.0	4.57 (m)
	β	78.2	3.75 (m)	78.6	3.75 (m)	78.5	3.68 (br d, 10.1)	78.2	3.76 (m)	78.6	3.82 (m)	78.3	3.77 (m)
	γ	30.1	1.98 (m)	30.1	1.98 (m)	30.2	1.82 (m)	29.9	1.98 (m)	30.1	1.95 (m)	30.0	1.96 (m)
	δ	a 20.5	a 1.18 (d, 6.3)	a 20.4	a 1.10 (d, 6.5)	a 19.6	a 1.01 (d, 6.7)	a 20.5	a 1.11 (d, 6.7)	a 20.3	a 1.10 (d, 6.1)	a 20.2	a 1.15 (d, 6.7)
		b 18.6	b 0.89 (d, 6.8)	b 18.7	b 0.92 (d, 7.3)	b 19.2	b 0.93 (d, 6.7)	b 18.5	b 0.88 (d, 6.7)	b 18.6	b 0.91 (d, 6.7)	b 18.5	b 0.90 (d, 6.7)
	CO	170.2		170.6		171.2		170.2		170.6		170.1	
	NH		7.26 (d, 8.0)		7.37 (d, 8.0)		8.16 (d, 9.8)		7.51 (d, 7.3)		7.29 (d, 7.9)		7.18 (m)
	β-OH		6.84 (d, 4.4)		6.32 (d, 4.4)		4.96 (d, 3.7)		7.07 (d, 4.9)		6.05 (d, 4.9)		7.08 (d, 4.3)
β-HyLeu-2	α	46.8	5.36 (d, 9.7)	46.5	5.57 (d, 9.8)	46.2	5.34 (d, 9.2)	46.2	5.39 (d, 9.8)	47.1	5.60 (d, 9.6)	46.8	5.43 (m)
	β	77.1	5.32 (d, 10.3)	78.7	5.11 (m)	77.1	5.07 (d, 7.9)	76.6	5.20 (d, 9.2)	79.0	5.22 (d, 10.4)	77.0	5.38 (d, 9.2)
	γ	30.6	1.73 (m)	30.0	1.71 (m)	30.8	1.78 (m)	30.7	1.76 (m)	29.9	1.74 (m)	29.9	1.74 (m)
	δ	a 19.5	a 1.10 (d, 7.0)	a 19.4	a 1.14 (d, 6.0)	a 18.9	a 0.95 (d, 6.7)	a 19.5	a 1.09 (d, 6.7)	a 19.4	a 1.17 (d, 6.7)	a 19.5	a 1.10 (d, 6.1)
		b 18.2	b 0.84 (d, 6.8)	b 18.4	b 0.84 (d, 6.8)	b 18.4	b 0.86 (d, 6.7)	b 18.4	b 0.86 (d, 6.7)	b 18.4	b 0.85 (d, 6.1)	b 18.2	b 0.86 (d, 5.5)
	CO	171.3		172.6		172.4		172.1		173.2		172.1	
	NH		6.77 (d, 9.7)		6.66 (d, 9.8)		9.17 (d, 9.2)		7.05 (d, 9.8)		6.64 (d, 9.6)		6.77 (d, 9.8)
N,*O*-Me_2Thr	α	64.4	4.05 (d, 10.3)	64.8	4.20 (d, 9.8)	65.2	4.24 (d, 10.4)	64.8	4.15 (d, 9.8)	64.7	4.21 (d, 10.4)	64.6	4.09 (d, 9.8)
	β	72.3	3.77 (m)	71.7	3.85 (m)	71.5	3.59 (m)	72.3	3.74 (m)	71.7	3.89 (m)	72.3	3.76 (m)
	γ	16.4	1.19 (d, 5.4)	16.4	1.26 (d, 5.9)	16.5	1.18 (d, 6.1)	16.4	1.20 (d, 6.1)	16.3	1.27 (d, 6.1)	16.4	1.20 (d, 6.1)
	CO	166.3		166.1		166.0		166.3		166.0		166.2	
	NMe	28.9	2.68 (s)	28.9	2.93 (s)	28.1	2.13 (s)	28.9	2.74 (s)	28.9	3.00 (s)	29.1	2.69 (s)
	OMe	57.1	3.41 (s)	57.0	3.45 (s)	57.0	3.36 (s)	57.1	3.42 (s)	57.0	3.47 (s)	57.1	3.42 (s)
Acetyl-2	1	171.6		170.6		170.2		171.4		170.6		171.5	
	2	22.4	2.27 (s)	22.6	2.14 (s)	22.5	2.17 (s)	22.4	2.24 (s)	22.5	2.14 (s)	22.4	2.27 (s)
Thr	α	52.8	5.02 (dd, 9.8, 1.4)	54.9	5.01 (dd, 8.8, 1.9)	55.0	5.13 (dd, 9.8, 1.8)	53.1	4.98 (m)	55.0	5.02 (m)	53.2	5.01 (m)
	β	69.6	5.50 (m)	75.0	5.35 (m)	74.4	5.01 (m)	70.2	5.44 (m)	74.9	5.41 (m)	70.0	5.53 (m)
	γ	16.7	1.22 (d, 6.8)	16.5	1.32 (d, 5.8)	16.4	1.33 (d, 6.1)	16.9	1.23 (d, 6.1)	16.5	1.33 (d, 6.1)	16.8	1.23 (d, 6.7)
	CO	169.0		167.9		168.5		168.5		168.8		168.8	
	NH		7.64 (d, 9.8)		7.64 (d, 8.8)		7.85 (d, 9.8)		7.81 (d, 9.8)		7.35 (d, 8.6)		7.57 (d, 9.8)
Pla	1	167.9		167.7		168.9		166.8		168.6		168.8	
	2	72.7	5.21 (dd, 8.8, 3.9)	70.7	5.10 (zdd, 8.5, 4.4)	73.5	4.90 (dd, 9.8, 3.7)	72.5	5.01 (m)	70.9	5.02 (m)	73.5	5.13 (dd, 9.2, 2.4)
	3	36.6	a 3.12 (dd, 15.2, 3.9)	38.4	a 3.33 (dd, 12.7, 8.5)	37.0	a 3.04 (dd, 14.0, 9.8)	36.5	a 3.04 (m)	38.4	a 3.22 (m)	36.0	a 3.14 (dd, 14.7, 2.7)
			b 2.98 (dd, 15.2, 8.8)		b 2.78 (dd, 12.7, 4.4)		b 2.90 (dd, 14.0, 3.7)		b 2.90 (m)		b 2.88 (m)		b 2.82 (dd, 14.7, 9.2)
	4	136.0		134.6		135.4		136.1		134.2		136.5	
	5/9	129.5	7.24 (m)	130.0	7.18 (m)	129.4	7.19 (m)	129.7	7.21 (m)	129.5	7.18 (m)	129.4	7.19 (m)
	6/8	128.6	7.28 (m)	128.8	7.18 (m)	128.9	7.34 (m)	128.5	7.29 (m)	128.8	7.28 (m)	128.5	7.28 (m)
	7	127.0	7.24 (m)	127.8	7.28 (m)	127.6	7.32 (m)	126.9	7.22 (m)	127.5	7.24 (m)	126.8	7.21 (m)

Table 2 (*continued*)

Residue	Position	YM-254890 (**1**) in $CDCl_3$				YM-385780 (**5**) in $CDCl_3$				YM-385781 (**6**) in $CDCl_3$			
		Major conformer		Minor conformer		Major conformer		Minor conformer		Major conformer		Minor conformer	
		^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)	^{13}C	^{1}H (mult., J in Hz)
N-MeDha/	α	145.5		141.5		53.9	5.08 (q, 7.3)	61.5	3.40 (m)	54.3	4.47 (q, 6.7)	54.2	4.58 (m)
N-MeAla-1	β	107.0	a 5.34 (d, 1.5)	122.6	a 5.70 (d, 1.5)	14.8	1.41 (d, 7.3)	12.6	1.44 (d, 6.7)	16.0	0.60 (d, 6.7)	14.5	1.42 (d, 7.9)
			b 5.09 (d, 1.5)		b 3.70 (d, 1.5)								
	CO	164.0		162.1		170.9		169.5		170.1		172.6	
	NMe	36.3	3.19 (s)	37.5	3.03 (s)	30.5	3.02 (s)	37.1	3.13 (s)	30.3	2.74 (s)	30.8	3.07 (s)
Ala	α	45.9	4.91 (m)	46.1	5.10 (m)	47.6	4.79 (m)	46.7	4.93 (m)	45.3	4.98 (m)	45.9	4.77 (m)
	β	18.0	1.40 (d, 6.8)	18.9	1.30 (d, 5.4)	16.0	1.48 (d, 6.1)	18.2	1.34 (d, 5.5)	18.7	1.28 (d, 6.1)	17.8	1.38 (m)
	CO	172.7		172.4		172.9		173.0		172.5		172.9	
	NH		8.51 (d, 9.3)		7.70 (d, 9.7)		6.16 (d, 8.6)		7.84 (d, 9.8)		7.33 (m)		8.30 (d, 9.2)
N-MeAla-2	α	56.5	4.76 (q, 6.8)	56.0	4.80 (q, 6.3)	67.9	3.62 (q, 7.3)	56.4	4.94 (m)	56.0	4.71 (q, 6.7)	56.5	4.57 (m)
	β	14.2	1.40 (d, 6.8)	14.0	1.49 (d, 6.8)	16.2	1.79 (d, 7.3)	13.9	1.29 (d, 6.7)	14.0	1.46 (d, 6.7)	14.2	1.34 (m)
	CO	170.0		171.0		174.1		170.2		170.7		169.8	
	NMe	31.5	2.90 (s)	31.1	2.88 (s)	40.1	2.87 (s)	32.0	2.91 (s)	31.0	2.85 (s)	31.4	2.88 (s)

NH of Ala, α-H of Ala/NMe of *N*-MeAla, α-H of *N*-MeAla/NH of β-HyLeu, α-H of β-HyLeu/NMe of *N*,*O*-Me_2Thr). The significant NOESY correlation between Pla and *N*-MeDha of **4** was not observed, so that the *cis–trans* conformation of the Pla/*N*-MeDha amide bond was not determined. It was not determined whether the conformational differences between **4** and **1** were due to structural differences or to varying dissolution in different NMR solvents. For the major conformer of **5** in $CDCl_3$, the ROESY correlations (α-H of β-HyLeu-2/α-H of *N*,*O*-Me_2Thr; H-2 of acetyl-1/NH of β-HyLeu-1, H-2 of acetyl-2/NH of Thr, α-H of Pla/NMe of *N*-MeAla-1, α-H of Ala/NMe of *N*-MeAla-2) indicated the presence of one *cis*-amide bond (β-HyLeu-2/*N*,*O*-Me_2Thr) and four *trans*-amide bonds (acetyl-1/β-HyLeu-1, acetyl-2/Thr, Pla/*N*-MeAla-1, Ala/*N*-MeAla-2). The difference between **5** and **1** in the *cis–trans* conformation of the amide bond (Ala/*N*-MeAla-2) and the chemical shifts suggested that the conformation of **5** differed significantly from that of **1**.[3] The difference between major and minor conformers of **5** remained elusive due to either very weak or absent ROESY correlations (*N*-MeAla-1/Ala, *N*-MeAla-2/β-HyLeu-2). For the major conformer of **6** in $CDCl_3$, the ROESY correlations (α-H of β-HyLeu-2/α-H of *N*,*O*-Me_2Thr, α-H of Pla/α-H of *N*-MeAla-1, α-H of Ala/α-H of *N*-MeAla-2; H-2 of acetyl-1/NH of β-HyLeu-1, H-2 of acetyl-2/NH of Thr, α-H of *N*-MeAla-1/NH of Ala, α-H of *N*-MeAla-2/NH of β-HyLeu-2) indicated the presence of three *cis*-amide bonds (β-HyLeu-2/*N*,*O*-Me_2Thr, Pla/*N*-MeAla-1, Ala/*N*-MeAla-2) and four *trans*-amide bonds (acetyl-1/β-HyLeu-1, acetyl-2/Thr, *N*-MeAla-1/Ala, *N*-MeAla-2/β-HyLeu-2). The minor conformer of **6** differed from the major conformer only in the conformation of the Pla/*N*-MeAla-1 amide bond, indicating that the minor conformer of **6** has the same conformation as the major conformer of **1**, whereas the major conformer of **6** has the same amide bond conformations as the minor conformer of **1**.

In order to evaluate the $G\alpha_{q/11}$ inhibitory activity of novel YM-254890 analogues **2**–**4** and semi-synthetic derivatives **5**–**6**, we examined their effects on 2MeSADP-induced Ca^{2+} influx activity in $P2Y_1$–$C_{6\text{-}15}$ cells in comparison with **1** (Table 3). Compounds **2** and **3** showed almost the same IC_{50} values as **1**, suggesting that there was some steric allowance around the acyl group of β-HyLeu-1. The inhibitory activity of **4** was about 40-fold less potent than that of **1**, indicating that the ab-

Table 3. Effect of YM-254890 analogues and semi-synthetic derivatives (**1**–**6**) on 2MeSADP-induced Ca^{2+} influx in $P2Y_1$–$C_{6\text{-}15}$ cells and ADP-induced platelet aggregation in human platelet-rich plasma

Compound	IC_{50} (μM, mean ± SE, $n = 3$)	
	2MeSADP-induced Ca^{2+} influx	ADP-induced platelet aggregation
1	0.20 ± 0.042	0.26 ± 0.082
2	0.23 ± 0.035	0.27 ± 0.13
3	0.19 ± 0.047	0.29 ± 0.12
4	7.4 ± 1.3	3.4 ± 0.37
5	9.3 ± 0.40	3.3 ± 0.66
6	0.22 ± 0.026	0.51 ± 0.16

sence of an acyl β-HyLeu residue was not tolerated by the $G\alpha_{q/11}$ protein. Interestingly, among the two YM-254890 hydrogenated diastereomers, which were derivatives of **1**, **6** showed almost the same IC_{50} values as **1**. This suggests that *N*-MeDha is not essential to the activity and that YM-254890 interacts with the $G\alpha_{q/11}$ protein without covalent modification by Michael addition. On the other hand, the other diastereomer **5** had significantly reduced activity. The conformations of **1**, **5**, and **6** in the assay buffer could not be examined because of the low solubility in water. However, the conformation of **5** in $CDCl_3$ differs from that of **1** whereas the conformations of **6** and **1** are similar, indicating that the conformational difference may play a critical role in determining activity. The inhibitory activity on ADP-induced platelet aggregation was also examined. Similar to 2MeSADP-induced Ca^{2+} influx activity in $P2Y_1$–$C_{6\text{-}15}$ cells, the inhibitory activities of **2**, **3**, and **6** on ADP-induced platelet aggregation were comparable to that of **1** whereas **4** and **5** exhibited significantly less activity.

3. Conclusion

We have discovered three novel YM-254890 analogues **2**–**4** from the fermentation broth of *Chromobacterium* sp. QS3666 and synthesized two hydrogenated derivatives **5**–**6**. Their structures were determined by one- and two-dimensional NMR studies and mass spectrometry. The structure–activity relationship was examined by comparing their $G\alpha_{q/11}$ inhibitory activity with that of **1**. This information could be helpful in achieving an understanding of the $G\alpha_{q/11}$ inhibitory mechanism of YM-254890 and the development of a therapeutic agent for the treatment of $G\alpha_{q/11}$-mediated diseases.

4. Experimental

4.1. General

Reagents and solvents were obtained from commercial suppliers and used as received. (2*S*,3*R*)- and (2*R*,3*S*)-β-HyLeu (2-amino-3-hydroxy-4-methylpentanoic acid) were obtained from Kanto Chemicals. (2*S*,3*R*)- and (2*R*,3*S*)-*N*,*O*-Me_2Thr were synthesized as described by Taniguchi et al.[4] 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 ^1H 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.

4.2. Fermentation

The strain 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%, $CaCO_3$ 0.4% (pH 7.0). After incubation at 28 °C for 3 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% (pH 6.5). The fermentation was carried out at 24 °C for 3 days with agitation of 250–400 rpm and aeration of 1 vvm.

4.3. Isolation

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 provide a brown extract (22.9 g). This oily extract was subjected to ODS flash chromatography (YMC-GEL ODS-A 120-S150, 100 × 60 mm, YMC) eluting 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 then subjected to silica gel flash chromatography (Kieselgel 60 0.040–0.063 mm, 60 × 130 mm, MERCK), eluting using a step gradient of a $CHCl_3$–MeOH solvent system to give seven fractions (F1–F7). Fractions F1 and F3 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 23.2 min were concentrated in vacuo and crystallized from *n*-hexane to afford YM-254891 (**2**) (287 mg). The fractions that eluted at 25.2 min were evaporated to dryness (56 mg), and the residue was purified by preparative HPLC (CAPCELL PAK C18 UG120, 20 × 250 mm, Shiseido; MeOH/H_2O (72:28); 8 mL/min; retention time 38.0 min) to yield YM-254892 (**3**) (36 mg). Fraction F4 was evaporated to dryness (880 mg), and the residue was successively purified by preparative HPLC (CAPCELL PAK C18 UG120, 20 × 250 mm, Shiseido; MeOH/H_2O (72:28); 8 mL/min; retention time 17.6 min), LH-20 column chromatography (Sephadex LH-20, 20 × 430 mm, Pharmacia Biotech; MeOH), preparative HPLC (YMC-PAK ProC18, 20 × 250 mm, YMC; MeCN/H_2O (50:50, 0.05% TFA); 10 mL/min; retention time 14.2 min), and preparative HPLC (YMC-PAK ProC18, 20 × 250 mm, YMC; MeCN/H_2O (50:50); 10 mL/min; retention time 14.4 min) to yield YM-280193 (**4**) (35 mg).

4.3.1. YM-254891 (2). White powder; $[\alpha]_D^{25}$ −72.9° (*c* 0.30, MeOH); UV (MeOH) end absorption; IR *ν*max (KBr) 3430, 3330, 2980, 2940, 1750, 1680, 1640, 1530, 1460, 1410, 1380, 1280, 1240, 1210, 1170, 1100, 1070 cm^{-1}; positive FABMS *m*/*z* 974 $[M+H]^+$, *m*/*z* 996

$[M+Na]^+$; negative FABMS m/z 972 $[M-H]^-$; HRFABMS m/z 974.5096 $[M+H]^+$, calcd for $C_{47}H_{72}N_7O_{15}$, $\Delta + 0.9$ mmu; ^1H and ^{13}C NMR are listed in Table 1.

4.3.2. YM-254892 (3). White powder; $[\alpha]_D^{25}$ −65.8° (*c* 0.30, MeOH); UV (MeOH) end absorption; IR νmax (KBr) 3430, 3340, 2970, 2940, 1750, 1680, 1640, 1530, 1460, 1410, 1380, 1280, 1220, 1160, 1100, 1070 cm^{-1}; positive FABMS m/z 1006 $[M+H]^+$, m/z 1028 $[M+Na]^+$; negative FABMS m/z 1004 $[M-H]^-$; HRFABMS m/z 1006.4795 $[M+H]^+$, calcd for $C_{47}H_{72}N_7O_{15}S$, $\Delta - 1.2$ mmu; ^1H and ^{13}C NMR are listed in Table 1.

4.3.3. YM-280193 (4). White powder; $[\alpha]_D^{25}$ −61.3° (*c* 0.30, MeOH); UV (MeOH) end absorption; IR νmax (KBr) 3430, 2980, 2940, 1750, 1650, 1540, 1460, 1410, 1380, 1310, 1280, 1220, 1160, 1090, 1070 cm^{-1}; positive FABMS m/z 789 $[M+H]^+$, m/z 811 $[M+Na]^+$; negative FABMS m/z 787 $[M-H]^-$; HRFABMS m/z 789.4031 $[M+H]^+$, calcd for $C_{38}H_{57}N_6O_{12}$, $\Delta - 0.3$ mmu; ^1H and ^{13}C NMR are listed in Table 1.

4.4. Hydrogenation of YM-254890 (1)

To a solution of **1** (30 mg, 0.03 mmol) in MeOH (5 mL) was added 10% Pd/C (12 mg), and the mixture was stirred under a H_2 atmosphere at room temperature for 4 h. The reaction mixture was filtered through Celite® and evaporated to dryness (29.8 mg). The residue was 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 to give 10 mg of **5** (34.7%) and 12 mg of **6** (41.6%).

4.4.1. YM-385780 (5). White powder; $[\alpha]_D^{25}$ −36.5° (*c* 0.20, MeOH); UV (MeOH) end absorption; IR νmax (KBr) 3420, 3330, 2960, 2940, 1750, 1650, 1540, 1450, 1380, 1280, 1230, 1160, 1100, 1070, 1030 cm^{-1}; positive ESIMS m/z 962 $[M+H]^+$, m/z 984 $[M+Na]^+$; HRFABMS m/z 962.5076 $[M+H]^+$, calcd for $C_{46}H_{72}N_7O_{15}$, $\Delta - 1.1$ mmu; ^1H and ^{13}C NMR are listed in Table 2.

4.4.2. YM-385781 (6). White powder; $[\alpha]_D^{25}$ −63.5° (*c* 0.20, MeOH); UV (MeOH) end absorption; IR νmax (KBr) 3430, 3330, 2970, 2940, 1750, 1640, 1530, 1450, 1380, 1280, 1230, 1170, 1100, 1070 cm^{-1}; positive ESIMS m/z 962 $[M+H]^+$, m/z 984 $[M+Na]^+$; HRFABMS m/z 962.5092 $[M+H]^+$, calcd for $C_{46}H_{72}N_7O_{15}$, $\Delta + 0.6$ mmu; ^1H and ^{13}C NMR are listed in Table 2.

4.5. Marfey's analysis of amino acids

Each of the YM-254890 analogues and semi-synthetic derivatives (1 mg) was dissolved in 6 N HCl (1 mL), heated in a sealed tube at 110 °C for 6 h, and the reaction mixture extracted with EtOAc (1 mL × 3). The aqueous layer was dried under Ar, and the residue dissolved in water (200 μL). Subsequently, this solution (20 μL) was treated with 1% FDAA solution (40 μL) in acetone and 1 M $NaHCO_3$ (8.5 μL), and heated at 40 °C for 1 h. After cooling to room temperature, 1 N HCl (20 μL) was added and the solution was analyzed by LC/MS (Alliance HT/ZQ (ESI), Waters) on a Cadenza CD-C18 (4.6 × 250 mm, Imtakt) at a flow rate of 1 mL/min at 40 °C, using a linear gradient from MeCN/H_2O (20:80, 0.1% formic acid) to MeCN/H_2O (45:55, 0.1% formic acid) over 30 min. The FDAA derivative of each acid hydrolysate was identified by comparing the retention times with those of authentic amino acids. The retention times of the FDAA derivative of the acid hydrolysate were as follows: L-Ala 13.1 min (D-Ala 15.9 min), L and L-allo-Thr 9.4 min (D-Thr 12.2 min, D-allo-Thr 10.6 min), L and D-*N*-MeAla 15.4 min, (2*S*,3*R*)-β-HyLeu 16.2 min ((2*R*,3*S*)-β-HyLeu 22.1 min, (2*S*,3*S*)-β-HyLeu 15.4 min, (2*R*,3*R*)-β-HyLeu 19.3 min), and (2*S*,3*R*)-*N*,*O*-Me_2Thr 17.9 min ((2*R*,3*S*)-*N*,*O*-Me_2Thr 19.2 min, (2*S*,3*S*)-*N*,*O*-Me_2Thr 20.2 min, (2*R*,3*R*)-*N*,*O*-Me_2Thr 21.8 min). The retention times of the FDAA derivatives of (2*S*,3*S*)-β-HyLeu, (2*R*,3*R*)-β-HyLeu, (2*S*,3*S*)-*N*,*O*-Me_2Thr, and (2*R*,3*R*)-*N*,*O*-Me_2Thr were determined using those of α-carbon racemized (2*R*,3*S*)-β-HyLeu, (2*S*,3*R*)-β-HyLeu, (2*R*,3*S*)-*N*,*O*-Me_2Thr, and (2*S*,3*R*)-*N*,*O*-Me_2Thr, respectively.[6] The configurations of Thr and *N*-MeAla could not be determined by the solvent system described above. The isocratic solvent system of MeOH/H_2O (40:60, 0.1% formic acid) was used to separate the FDAA derivatives of Thr and *N*-MeAla. The retention times of the FDAA derivative of the acid hydrolysate were as follows: L-Thr 7.7 min (D-Thr 13.4 min, L-allo-Thr 8.8 min, D-allo-Thr 10.3 min) and L-*N*-MeAla 19.7 min (D-*N*-MeAla 19.1 min).

4.6. Racemization of amino acids[6]

To a solution of amino acid (3 mg) in water (200 μL) were added triethylamine (80 μL), and acetic anhydride (80 μL). After stirring at 60 °C for 1 h, the reaction mixture was evaporated to dryness. The residue was dissolved in 6 N HCl (400 μL), and the solution was stirred at 110 °C for 12 h and then evaporated in vacuo to give an α-carbon racemized amino acid.

4.7. Chiral HPLC analysis

Each of the YM-254890 analogues and semi-synthetic derivatives (1 mg) was dissolved in 6 N HCl (1 mL) and heated in a sealed tube at 110 °C for 6 h. The reaction mixture was then extracted with EtOAc (1 mL × 3). The organic layer was dried under Ar, and the residue dissolved in MeOH (200 μL). 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 at 30 °C with UV detection at 254 nm, using 2.0 mM $CuSO_4$ in 2-propanol/H_2O (15:85) as an eluent. The retention times of the acid hydrolysates of all YM-254890 analogues and derivatives were 36.0 min (D-Pla; 27.9 min L-Pla).

5. Bioassays

5.1. Intracellular calcium mobilization

$C_{6\text{-}15}$ cells transfected with human $P2Y_1$ receptor cDNA were seeded into 96 well black clear tissue culture plates 24 h before assay. The cells were loaded for 1 h at 37 °C with 4 μM Fluo-3, AM (Molecular Probes) in Dulbecco's Modified Eagle Medium containing 10% FBS and 2.5 mM probenecid (Sigma). After washing with Hanks balanced salt solution (GIBCO BRL) and 20 mM HEPES containing 2.5 mM probenecid, transient changes in intracellular calcium concentration caused by 2MeSADP (2-methylthioadenosine 5′-diphosphate) were monitored using the FLIPR system (Molecular Devices). A test sample was added 5 min before the addition of 2MeSADP. Data were obtained using the maximum fluorescence counts after addition of the agonist.

5.2. Platelet aggregation

Platelet-rich plasma was prepared by centrifugation of citrate-anti-coagulated blood (3.8% sodium citrate–blood = 1:9) from healthy human volunteers who had not been on any medication for 2 weeks preceding the experiments. Platelet aggregation in human platelet-rich plasma was measured using an aggregometer (Hema Tracer 212, MC Medical) by recording the increase in light transmission through a stirred suspension maintained at 37 °C for 7 min. Platelet aggregation in platelet-rich plasma ($3 \times 10^5/\mu L$) was induced by ADP (5 μM). Inhibition was calculated by dividing the maximum rate of decrease in absorbance of a mixture containing the test sample by the maximum rate in the buffer control.

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References and notes

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